

A PHYTOCHEMICAL AND
PHARMACOLOGICAL STUDY OF *Acalypha*
wilkesiana var. *macafeana* hort.
(EUPHORBIACEAE JUSS.): ANTIOXIDANT
AND ANTIBACTERIAL ANALYSES

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ABSTRACT

A tropical shrub from Euphorbiaceae (Juss.) family, namely *Acalypha wilkesiana* var. *macafeana* hort. was investigated for its antioxidant and antibacterial properties. The antioxidant properties were assayed by Ferric Reducing Antioxidant Power (FRAP) assay, 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay and β -carotene bleaching assay. Assessment of its antibacterial properties was conducted with pour plate disc diffusion assay and dilution methods. The plant was collected, dried, grinded and soaked continuously in four different solvent starting from non-polar to polar solvent: hexane, ethyl acetate, ethanol and water. The crude extracts were concentrated under pressure and kept in -20 °C prior to investigation.

The ethanol extract of *A. wilkesiana* var. *macafeana* hort. exhibited good antioxidant and antibacterial activities with results more potent than the standards used. To further locate the bioactive constituents of the plant, we fractionated the ethanol extract leading to five fractions, namely F1, F2, F3, F4 and F5. Both antioxidant and antibacterial assays were conducted upon all the five fractions. Results showed profound activity from F5 of both antioxidant and antibacterial properties which warrants further investigation.

To shed light on the active constituents of F5, identification was done with high performance liquid chromatography (HPLC), liquid chromatography mass spectrometry (LCMS) and nuclear magnetic resonance (NMR). Separation was obtained with reversed phase HPLC which showed one major compound and 6 minor compounds. The major compound was collected with a fraction collector and identified as geraniin via interpretation and comparison of its NMR shifts, while the other 3 compounds were identified by fragmentations of LC-MS. The compounds identified are β -glucogallin, potentillin and sanguin H-6. All identified compounds are ellagitannins, except for β -glucogallin which is a gallotannin.

The *in vitro* cell-based assay was performed to HepG2 cells to assess the ability of antioxidants like ellagitannins to protect cells against oxidative insults, and F5 was observed to be able to protect cells against cell death induced by *t*-BHP insults in a dose-dependent manner. F5 was also found non-toxic in the concentration needed to protect the cells, which is 100 μ g/mL.

We then explored the synergistic property of the tannin fraction, F5 with commercial antibiotics and observed F5 and ampicillin inhibit the growth of *Staphylococcus aureus* synergistically. Field Emission Scanning Electron Microscopy (FESEM) analysis was able to demonstrate that the bactericidal mechanism of F5 involves cell wall lysis as the result illustrates indentation of the cell surface and some showed total collapse of the cells. To explore its ability to be formulated and used as a topical agent for treating bacterial infections, a preliminary formulation was made incorporating F5, and formulated with 3 different bases. The formulation made with the paraffin base was observed able to exert the antibacterial property of F5 against *Staphylococcus aureus* in the *in-vitro* assay. *In vivo* animal study on guinea pigs with an incised cut infected with *Staphylococcus aureus* and treated with the formulation showed that the closure and healing of the wound was faster than Burnol[®].

Our results indicate possible use of ellagitannins from *A. wilkesiana* var. *macafeana* hort. with ampicillin to treat *Staphylococcus aureus* infections as it is bactericidal via a mechanism involving cell lysis. It also illustrates the possibility to be used as a topical wound healing agent with respect to its antibacterial and antioxidant properties. Ellagitannins from *A. wilkesiana* var. *macafeana* hort. can be viewed as a possible bactericidal agent that can contribute to the development of topical antibacterial drug or cosmetics in tropical countries.

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LIST OF PUBLICATIONS

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GLOSSARY OF ABBREVIATIONS

<i>g</i>	Gram
<i>h</i>	Hour
<i>min</i>	Minutes
<i>L</i>	Liter
<i>mL</i>	Mililiter
μL	Microliter
<i>s</i>	Singlet
<i>d</i>	Doublet
<i>m</i>	Multiplet
<i>dd</i>	Double doublet
$^1\text{H-NMR}$	Proton Nuclear Magnetic Resonance
$^{13}\text{C-NMR}$	Carbon-13 Nuclear Magnetic Resonance
<i>MS</i>	Mass Spectrometry
<i>TOF</i>	Time of Flight
<i>TLC</i>	Thin Layer Chromatography
<i>HPLC</i>	High Performance Liquid Chromatography
<i>UV</i>	Ultraviolet
<i>DPPH</i>	2,2-diphenyl-1-picrylhydrazyl
<i>FRAP</i>	Ferric Reducing Antioxidant Power
<i>HepG2</i>	Human cancer cell line
<i>m/z</i>	Mass to charge ratio

CHAPTER 1

INTRODUCTION

1.1 General

People have been using plants for healing since centuries ago, as parts of powders, decoctions, or potions and herbal drinks. The ethnobotanical study leads researches to explore the medicinal plants used by the natives to understand how it works, improve the preparations used and finding a standard remedy. The research then extends to fingerprinting the plant extracts, identifying the bioactive secondary metabolites substances in the plants, knowing their relative contribution to the effects of the extracts and identifying toxicological doses of the extracts. The earliest isolation of an active compound from plants would be the isolation of morphine from *Papaver somniferum* L. (opium) in the early 19th century (Kinghorn 2001; Balunas & Kinghorn 2005). Other isolations from medicinal plants that have been discovered in the earlier years were cocaine from *Erythroxylum coca* Lam., codeine from *Papaver somniferum* L., digitoxin from *Digitalis sp.*, and quinine from *Chincona sp.* which some are still in use until now (Newman et al., 2000; Butler 2004). Importance of natural products are seen by scientists in most health related and medicine aspects from as simple as ageing to epilepsy, from

obesity to cardiovascular diseases, from healthy lifestyle to treatment of diabetes. All portrays the significance of natural products as a source of nutrition to human wellness.

Research on chemical and biological properties of natural products over the past two centuries has resulted in the discovery of many drugs for the treatment of human diseases. More than 50% of all the drugs currently in use are of natural product origin (Balandrin 1993). Higher plants have been the source of medical agents since earliest time and continue to play a dominant role in the health care industry until now (Farnsworth 1985). Secondary metabolites are the compounds responsible in plants bioactive properties. Biosynthetic pathways are responsible for the occurrence of both the primary metabolites and secondary metabolites in plants. Primary metabolites are any intermediate in, or product of primary metabolism, which are metabolic activities that are common in most living cells and are necessary for growth, maintenance and survival (Attwood & Cammack 2006) for example carbohydrates and protein. Secondary metabolites are traditionally defined as substances that appear to have no explicit role in the internal economy of the organism that produces it (Williams et al., 1989) and will be considered as a bioactive compound when it exerts biological effect on other organism. Others define secondary metabolites as a compound whose biosynthesis is restricted to selected plant groups and are

synthesized to address specific needs, for example floral scent and pigments from flowers attract pollinators and enhance fertilization (Pichersky & Gang 2000).

Identifying the bioactive secondary metabolites in traditionally used plants is becoming an interest to more and more researchers worldwide. The research is expanding as substantial numbers of structures are being identified from plants. These compounds are then screened for their biological activity to assess its bioactive properties. Chemical constituents from plants can be classified based on their biosynthetic origins, where they are divided into 3 major group i.e., terpenoids which are derived from isopentylidiphosphate (IPP), alkaloids which are biosynthesized from amino acids and contain nitrogen and phenolic compounds which are formed by either the shikimic acid pathway or the malonate/acetate pathway (Croteau et al., 2000).

Experimentation with single constituent provides better understanding of the mechanisms involved in a traditionally used plant or herbs. Without identifying its active compound, the mechanism of action of the plant or herb cannot be determined. In explaining the phytochemical basis of herbal medicines, herbalists are assessing plant properties in a holistic approach where the properties are given by the sum of totality of its constituents (Penggelly 2005).

Phytochemists are actively identifying and isolating active constituents from the plants and assessing their properties as a single constituent. Knowledge of individual constituents is essential to understand pharmacological activity, pharmacokinetics, potential toxicology and interactions with pharmaceutical drugs.

Scientists embarked on antibiotics in the 19th century, with sulfa drugs in the 30's (Drews 2000) and penicillin in the 40's (Ligon 2004). Since their introduction, antibiotics have allowed the treatment of bacterial infections and have saved many millions of lives (Fauci & Morens 2012). The discovery of penicillin by Fleming in 1928, as reported in the British Medical Literature was the beginning of the 'Golden age of antibiotics'. The structure was new to the chemistry world when it was first elucidated, but soon enough everything about it was revealed as research on penicillin continues. Penicillin was then semi-synthesized to derive the structure as resistance to the antibiotic was observed almost just after it was released and was used. The bacterium almost immediately produced protective enzymes and produces β -lactamases that were able to degrade penicillin. The search for antibacterials continues, as resistance become a more alarming problem and 'Mother Nature' still proves to become the most important source for finding new compounds for this purpose (Newman et al., 2000).

All antibiotics share an inherent weakness as the bacteria they target almost continuously develop new mechanisms of resistance (Davies & Davies 2010) on account of inadequate posology in hospital settings and communities (Buffet-Bataillon et al., 2012). Therefore, there is an obvious need to counteract bacterial resistance. One way to fight bacterial resistance is the use of multi component therapy. Indeed, combination between two or more antibiotics or natural products with antibiotics broadens bacterial targets or increase action on the same target (Wright 2012). Berdy et al. (2005) reported the presence of 200,000 bioactive natural products; therefore there is the exciting possibility to use natural products to maintain or boost the clinical effects of antibiotics via synergistic studies (Wiart 2012).

Important physiological functions involves normal cell metabolism. Normal metabolism produces oxidant by products (reactive oxygen species, ROS) to which causes damage to DNA, protein, and lipid which is the major contributor to aging and degenerative diseases (Ames et al., 1993). Endogenous sources of ROS would be from the perixosomes, mitochondria, cell activation and cytochrome P450 metabolism (Jatawa et al., 2012). Besides the endogenous sources of oxidants from metabolism, exogenous sources would also increase the load. Exogenous sources may come from oxides of

nitrogen in smoke, ultraviolet light, ionization radiation, environmental toxins and from metals and ions (Noh & Ha 2011).

Excess ROS leads to oxidative stress which is defined as the imbalance between antioxidants and oxidants in favour of the oxidants (Dudonne et al., 2009). The production of ROS and free radicals are normally accommodated by an elaborate endogenous antioxidant system. However, due to environmental, lifestyle, and other emerging factors, the elaborate system is abortive and causes excess of the radicals (Lima et al., 2007). Oxidative stress causes stress condition at cellular level thus, causing structural damage to the DNA, membrane, lipids, and proteins.

In fact, oxidative stress has been recognized to be involved in the diagnosis of several chronic diseases including cardiovascular diseases, cancer, diabetes, arthritis, nephropathy and the list continues on (Jatawa et al., 2012; Ames et al., 1993). It is also the major cause for minor illness, fatigue, infertility and aging. Studies have recognized the involvement of ROS in the etiology of liver diseases such as hepatitis, fibrosis, cirrhosis, and hepatocellular carcinoma (Vitaglione et al., 2005). Liver is a vital organ which plays a major role in metabolism including glycogen storage, plasma protein synthesis, hormone production and detoxification of the body. Consequently, mechanisms of

protection of liver are of high interest, and natural antioxidants as therapeutic agents are sought to counteract oxidative stress generally, and liver damage specifically.

As part of an ongoing study to identify antioxidants and antibacterial from medicinal plants (Wijaya et al., 2011a; Wijaya et al., 2011b), we have previously isolated flavanoid glucosides, flavanones and pyrrolizidine alkaloids. One of the largest group of flowering plants is the Euphorbiaceae with 300 genera and 7500 species (Wiart 2006) which provides numerous plants used for treatment of inflammatory conditions, wounds and bacterial infections. For example, *Acalypha racemosa* Wall. ex Baill. and *Acalypha indica* Linn. are used to treat liver disorders and hepatic dysfunction on probable account of its antioxidant properties (Iniaghe et al., 2008; Matthew et al., 2011). Other *Acalypha* species are also studied for their antioxidant and cytoprotective properties (Kavitha et al., 2009; Joy et al., 2010; Onocha et al., 2011a; Onocha et al., 2011b). An unidentified variety of *Acalypha wilkesiana* seeds are used as with a mixture of other herbs as treatment for breast cancer and inflammation (Bussing et al., 1999) and are found to be inducing apoptosis in U87MG (human glioma cells) and A549 (human lung carcinoma cells) by causing single stranded and double stranded DNA breaks (Lim et al., 2011).

Medicinal plant members of the family Euphorbiaceae impart anti-infective and wound healing properties (Wiart 2004). For instance, A bark extract of *Jatropha curcas* L. (Euphorbiaceae) which is traditionally used to treat diarrhoea and dysentery, inhibited the growth of *Bacillus subtilis*, *Sarcina lutea* and *Salmonella thyphi* with inhibition zone diameters of 18.0 ± 1.0 mm, 17.0 ± 0.5 mm and 17.0 ± 0.2 mm, respectively at a dose of 3 mg/disc (Gupta et al., 2011). *Sapium sebiferum* L. (Euphorbiaceae) is also used traditionally to treat diarrhoea, exhibited antimicrobial activity against Gram-negative *Escherichia coli* at a dose of 1.5 mg/mL (Chaudhary et al., 2011). An extract of *Acalypha indica* L. (Euphorbiaceae) inhibited Gram-positive bacteria and used traditionally used for bronchitis, pneumonia and asthma (Somchit et al., 2010). *Croton hirtus* L'Hér. and *Euphorbia hirta* L. (Euphorbiaceae) exhibited antibacterial activity against *Bacillus cereus*, *Bacillus subtilis* and *Staphylococcus aureus* (Wiart et al., 2004).

1.2 Aim and objectives of the study

The overall aim of this study was to identify active compound(s) from *Acalypha wilkesiana* var. *macafeana* hort. that elicits antioxidant, cytoprotective and antibacterial properties and examine their ability to be used in a topical formulation.

Objectives of the study are:

1. To collect *A. wilkesiana* var. *macafeana* hort. and extract its constituents with sequential maceration.
2. To screen the antioxidant properties of the extracts with ferric reducing antioxidant potential (FRAP) assay, DPPH radical scavenging assay and β -carotene bleaching assay.
3. To evaluate the cytotoxicity and cytoprotective concentrations of the active extract.
4. To screen the antibacterial properties of the extracts with the pour plate disc diffusion assay.

5. To determine the minimum inhibition concentration (MIC) and bactericidal concentration (MBC) of the active extract.
6. To evaluate the location of the active constituent(s) with fractionation by chromatographic means.
7. To isolate the major active constituent(s) with High Performance Liquid Chromatography (HPLC) and identify by spectroscopic means which includes Liquid Chromatography-Mass Spectrometry (LC-MS) and Nuclear Magnetic Resonance (NMR).
8. To produce a topical formulation from the active constituent(s) and test it for bacterial infection and wound healing properties.

CHAPTER 2

LITERATURE REVIEW

2.1 Natural Product Approach in Drug Discovery

Natural products were the first ever cure available to mankind in history as no synthetic medicines were yet discovered then (Ganesan 2008). Thus, plant natural product approach in drug discovery brings great importance with two premises; natural products interrogate a different area of chemical space than synthetic compounds, and natural products are amenable to further development (Ganesan 2008). Over the past few years, pharmaceutical companies have scaled down their funding and interests to natural products due to the drawbacks that are encountered in natural product research, which includes time consuming process of screening, isolation and structure elucidation of the compounds and challenging compound development (Butler 2004).

However recently, a renewed interest has flaunted as the challenges of natural product approach to drug discovery is overcome by improvement in technology, hence the continuous effort in drug discovery from natural products until now (Cragg et al., 2012). As to the year 2008, over 100 natural

product-derived compounds are currently undergoing clinical trials and 100 more are in preclinical development (Harvey 2008) of which anticancer and anti-infectives leads the list. Until now, still more natural product derived compounds serve as basic framework for design and synthesis of drugs (Cragg et al., 2012). The immense chemical complexity and diversity of natural product from plants represent therefore an important source of drugs. Researchers will continue to investigate flowering plants in high biodiversity ecological systems like the rainforest of Malaysia.

2.2 Plants in Malaysia

The Malaysian rainforest stores a large number of flowering plant species of which have been used by Orang Asli and the Malays as source of food and medicine (Wiar 2006). Indeed, about 10, 000 species of flowering plants and 2000 species of lower plants are available in Peninsular Malaysia, and only 16 % are claimed to be used traditionally (Latif et al., 1984). Common modes of administration of these plants by traditional Malaysians are oral and topical and are used as decoctions, infusions, poultices or simply pounded (Ong et al., 2011). The earliest record on the use of plants as medicine in Malaysia was reviewed by Burkill and Haniff (1930), Gimlett and Burkill (1930) and Burkill (1966) whereby several of them belongs to the family Euphorbiaceae Juss.

2.3 The Family Euphorbiaceae Juss.

The family Euphorbiaceae found by A.L. de Jussieu in 1789, or known as the Spurge family is a large family of flowering plants with 300 genera and around 7500 species which includes trees, shrubs and herbs (Wiart 2006a). Several characteristics in identifying species in this family include 3-locular capsules, stipules and spikes of tiny flowers (Nelson 1996). The family is divided to 4 subfamilies which are the Phyllanthoideae (ovules 2 per loculus; no latex), Euphorbiodeae (ovules 1 per loculus; latex usually present) in which includes the Acalypheae, Porantheroideae (ovules 2 per loculus; no latex) and the Ricinocarpoideae (ovules 1 per loculus, latex present) (Hickey & King 1988). Several members of the family Euphorbiaceae Juss. are used as traditional remedies (Table 2.1) (Ong 2006).

2.4 The Genus *Acalypha* L.

The genus *Acalypha* was first described by Linnaeus in 1753. As the third largest genus in the family Euphorbiaceae after *Euphorbia* L. and *Croton* L., *Acalypha* L. comprises of 450 species. *Acalypha* species are widespread geographically, with greatest diversity in the tropics and sub-tropics (Sagun et

al., 2010). As of a review from Sagun et al. (2010), from the 450 species of *Acalypha*, there are 28 species

Table 2.1. Plants used traditionally in Malaysia that belong to the Euphorbiaceae Juss. and their claimed medicinal purpose.

No.	Scientific Name	Vernacular Names	Traditional uses	References
1	<i>Sauropus androgynus</i> (L.) Merr.	“Cekur manis”, “katok”, star gooseberry	Postpartum relief and cardiovascular illness	Ong 2006; Koh et al., 2009
2.	<i>Phyllanthus emblica</i> L.	“Buah Melaka”, Indian gooseberry	Treatment of fever, headaches, migraine	Burkill 1966; Ong, 2006
3.	<i>Mallotus barbatus</i> Müll. Arg.	“Balik angin”	Treat abscesses and wounds, back and joints ache	Fatan Hamamah et al., 2003; Ong 2006
4.	<i>Bridelia insulana</i> Hance.	“Kenidai”	Treat itchness of skin	Ong 2006
5.	<i>Macaranga triloba</i> (Thunb.) Müll. Arg.	“Mahang merah”	Treatment of fungal and bacterial infections	Ong 2006; Lim et al., 2009
6.	<i>Aleurites mollucana</i> (L.) Willd.	“Buah keras”, candlenut	Treatment of constipations, gonorrhea and dysentery	Ong 2006
7.	<i>Acalypha wilkesiana</i> Müll. Arg.	“Akalifa”, Jacob’s coat, copper plant	Treatment of hemorrhoids, inflammation, skin infections	Ong 2006
8.	<i>Acalypha hispida</i> Burm. f.	“Ekor kucing”, Fox’s tail	Treatment of ulser, skin infections, asthma and flu	Ong 2006
9.	<i>Codiaeum variegatum</i> (L.) Blume.	“Pokok pudding”	Treatment of syphilis, abscesses, and fever	Ahmad & Holdsworth 2003; Ong 2006
10.	<i>Excoecaria cochinchinensis</i> Lour.	“Sambang darah”, Chinese croton	Post partum and menstrual pain relief	Ong et al., 1996; Ong 2006

recognized in the Malesian region which consists of Peninsular Malaysia, Borneo, Papua New Guinea, Indonesia and Philippines. Two species are exclusively West Malesian, 20 species are East Malesian, while six other species are distributed throughout the region (Sagun et al., 2010).

2.4.1 Botanical Features

The genus *Acalypha* L. consists of mainly trees and shrubs, which provides economical, ecological and ornamental plants (Govaerts et al., 2000). The description and taxonomy of the genus *Acalypha* L. has been attempted by various authors such as Levin (1998), Qin et al. (2006), Sagun and Levin (2007) and latest by Sagun et al. (2010). The species are either monoecious or dioecious.

The leaves are alternate, stipulate and serrate (Figure 2.1). The staminate flowers present 4 valvate sepals, 4 to 8 stamens and vermiform anthers. The pistillate flowers are bracteate with 3, 4 or 5 sepals and 2 to 3 carpels with one ovule per carpel (Figure 2.3). Many species of *Acalypha* L. share the characteristic of pistillate flowers and fruits. Majority of the species have in fruit accrescent (grows in size after flowering) pistillate bracts that vary in number and shape of the lobes (Figure 2.2). The fruits are bilocular or trilocular capsules (Figure 2.4). Stipules morphology vary from linear or needle like to broad ovate shape (Figure 2.5).

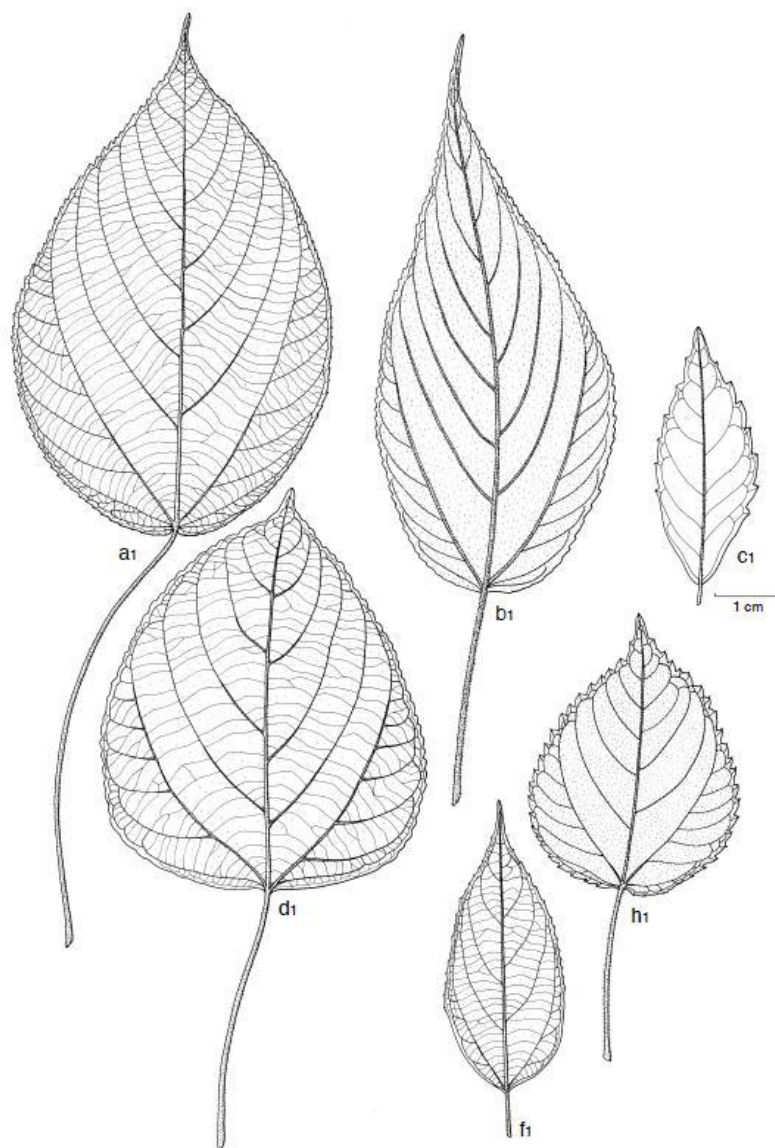


Figure 2.1. Leaf morphology of *Acalypha* L. sp. a1) *A. amentaceae* Roxb. b1) *Acalypha angatensis* Blanco. c1) *A. capillipes* Mull. Arg. d1) *A. cardiophylla* Merr. f1) *A. caturus* Blume. h1) *A. novoguineensis* Warb. (Sagun et al., 2010).

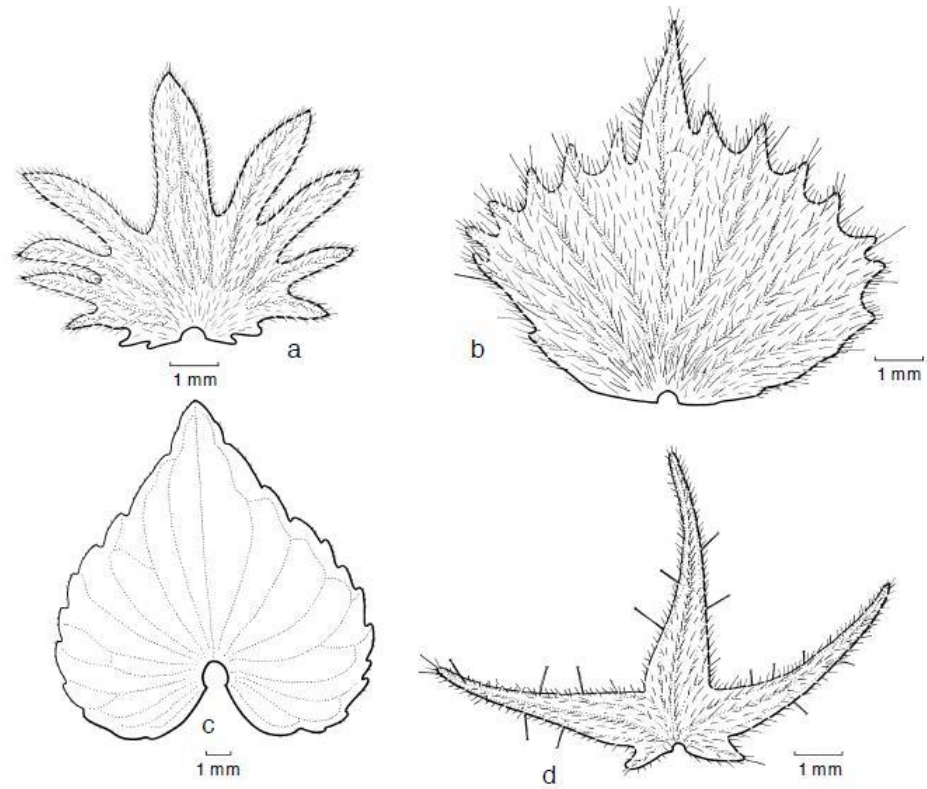


Figure 2.2. Pistillate bract morphology of *Acalypha* L. sp. a) *A. amentaceae* Roxb. b) *A. angatensis* Blanco. c) *A. australis* L. d) *A. brachystachya* Homem. (Sagun et al., 2010).

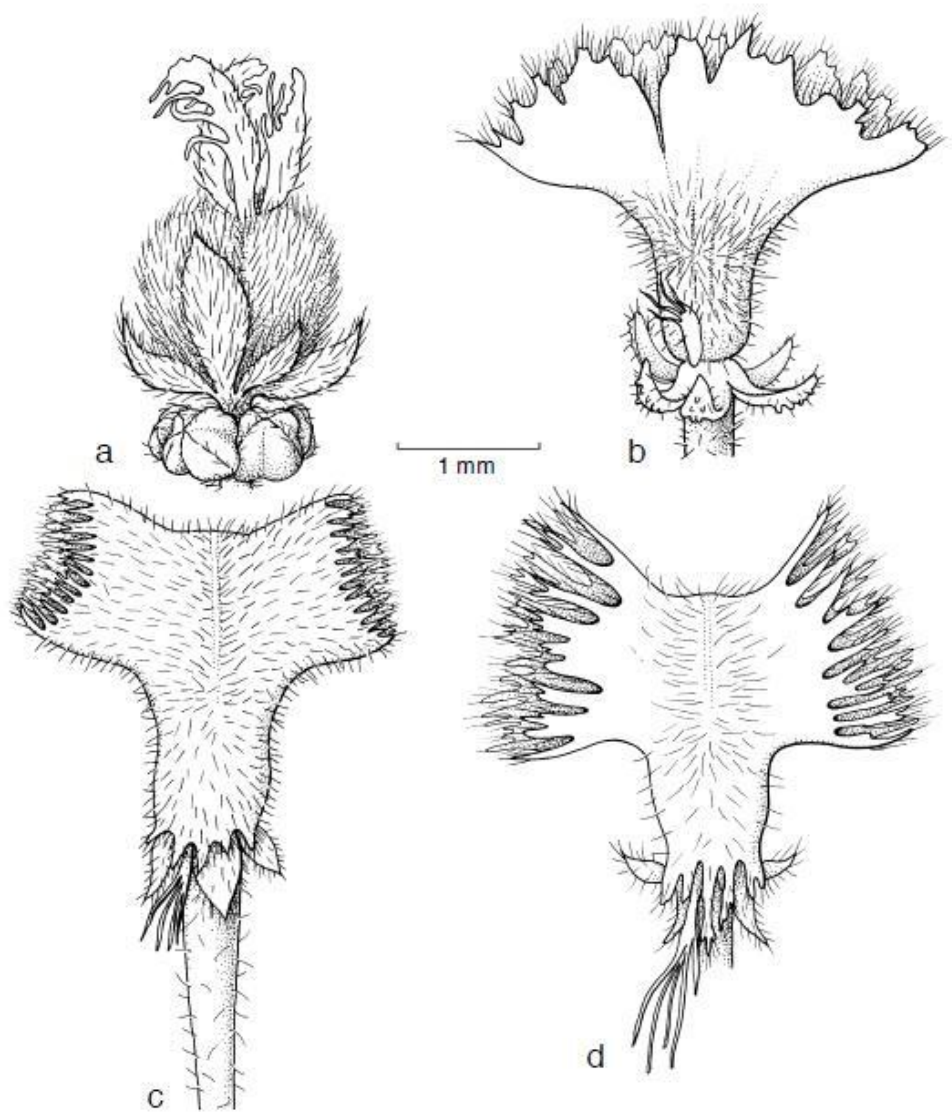


Figure 2.3. Allomorphic pistillate flowers of *Acalypha* L. sp. a) *A. antagensis* Blanco. b) *A. bracystachya* Homem. c) *A. indica* L. d) *A. lanceolata* Willd. (Sagun et al., 2010).

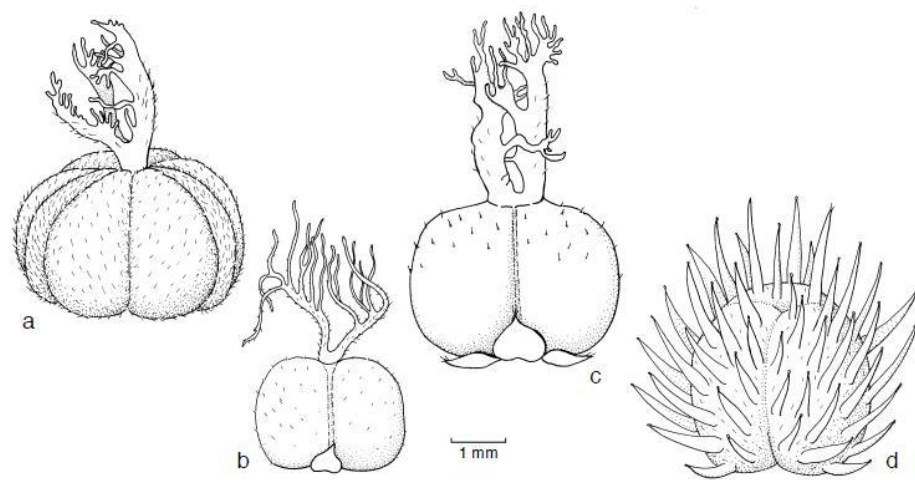


Figure 2.4. Fruit morphology of *Acalypha* L. sp. a) *A. cardiophylla* Merr. b) *A. cinnamomifolia* Pax & K.Hoffm. c) *A. caturus* Blume. d) *A. siamensis* Oliv ex Gage. (Sagun et al., 2010).

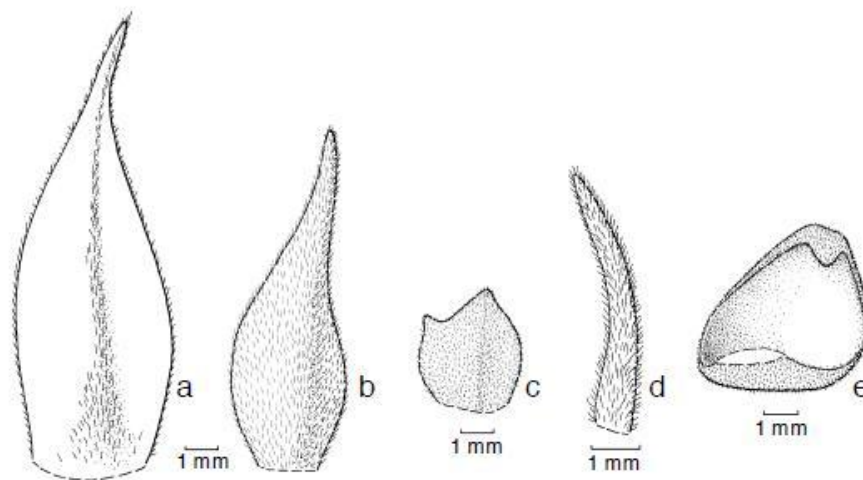


Figure 2.5. Stipule morphology of *Acalypha* L. sp. a) *A. amentaceae* Roxb. b) *A. angatensis* Blanco. c) *A. cardiophylla* Merr. d) *A. hellwigii* Warb. e) *A. longispica* Warb. (Sagun et al., 2010).

2.4.2 Pharmacological Properties

Several line of evidences points to the fact that members of the genus *Acalypha* L. are pharmacologically active (Solomon et al., 2005; Olundunmoye et al., 2006; Kambara et al., 2006; Mothana et al., 2008; Canales et al., 2011). Antibacterial and antifungal properties have been observed from *A. indica* L., *A. fruticosa* Forssk., *A. communis* Müll. Arg., *A. siamensis* Gagnep., *A. monostachya* Cav., *A. hispida* Burm. f. and *A. wilkesiana* Müll. Arg. (Solomon et al., 2005; Olundunmoye et al., 2006; Kambara et al., 2006; Mothana et al., 2008; Canales et al., 2011). Antioxidant and protective properties were elicited by *A. indica* L., *A. fruticosa* Forssk., and *A. racemosa* Wall. ex Baill. (Badami et al., 2007; Iniaghe et al., 2008; Mothana et al., 2008). Extracts of *A. wilkesiana* Müll. Arg., *A. alopecuroidea* Jacq. and *A. australis* L. displayed anti-cancer properties against different cell lines *in vitro* (Madlener et al., 2010; Shin et al., 2012).

In vivo studies evidenced the anti-diabetic properties from *A. wilkesiana* Müll. Arg. and *Acalypha indica* L. (Ikewuchi et al., 2011; Itankar et al., 2011) but the active constituents are yet unknown. A recent study from Quds et al. (2012) showed antiemetic property of *A. ornata* Hochst. ex A. Rich., *A. wilkesiana* Müll. Arg. and *A. fimbriata* Schumach. & Thonn.

2.4.3 Chemical properties

Oddly enough, the genus *Acalypha* L. has not drawn much attention to trace its active constituents, despite the fact that significant studies on the pharmacological properties have been undertaken. There is a dearth of information about the phytochemistry of this genus. Preliminary studies resulted in the identification of flavonoids, terpenes and tannins. Indeed, identification of compounds from *A. hispida* Burm. f. from two studies have isolated ellagitannins (geraniin, mallotusin, phyllantusin C, euphorbin A and B, acalyphidin M1, M2 and D1, furosin and reynadinin A) (Figure 2.6) and flavonoids (quercetin-3-O-rutinoside and kaempferol-3-O-rutinoside) from *Acalypha hispida* Burm. f. (Amakura et al., 1999; Adesina et al., 2000) (Figure 2.7).

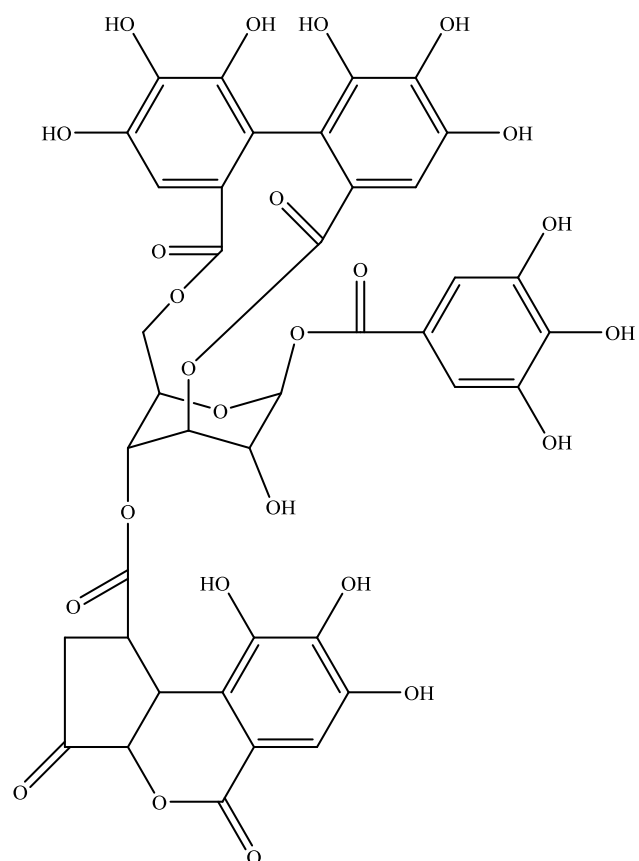


Figure 2.6 (a). Tannins isolated from *Acalypha* sp. - Acalyphidin M1

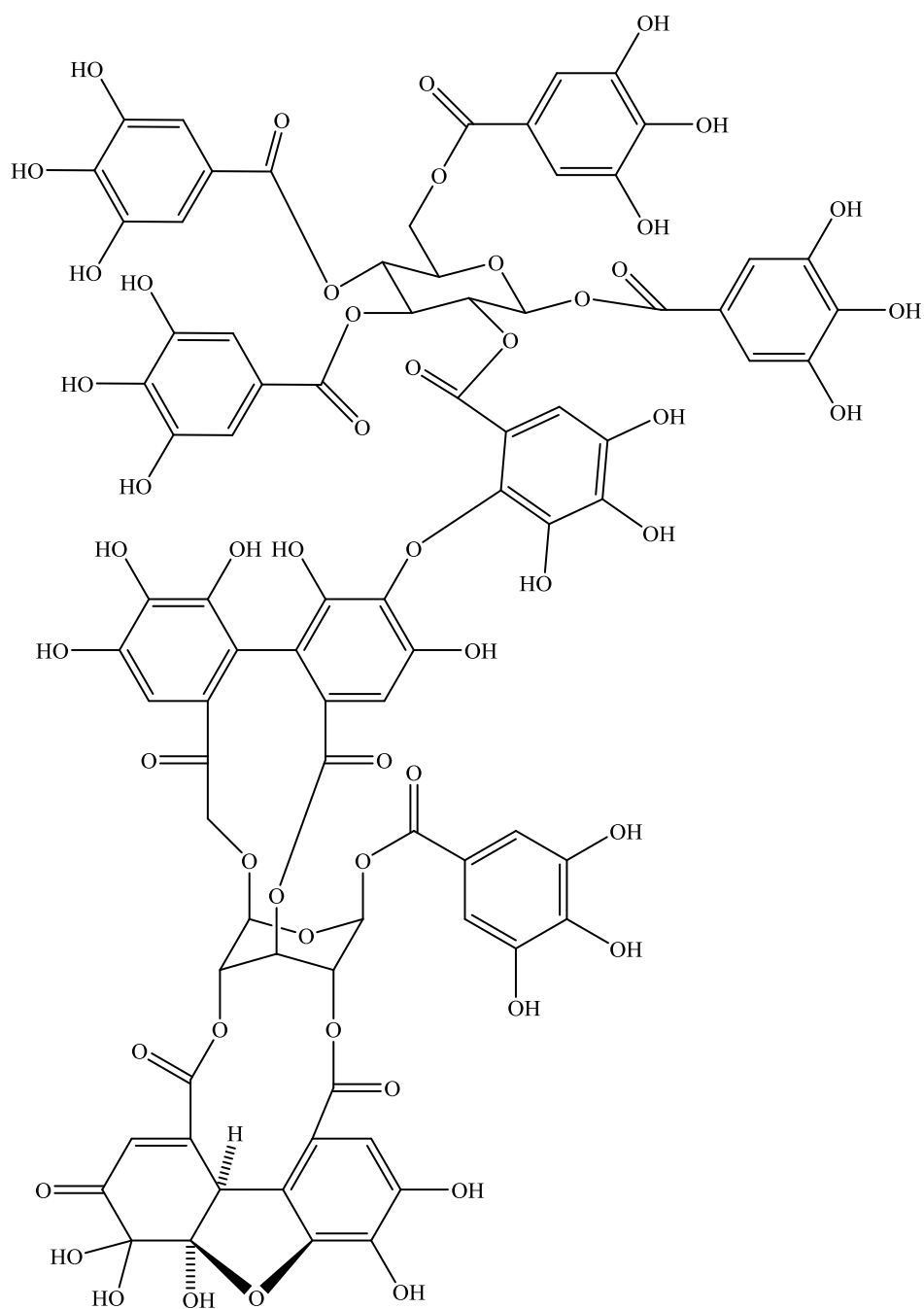


Figure 2.6 (b). Tannins isolated from *Acalypha* sp. - Euphorbin A

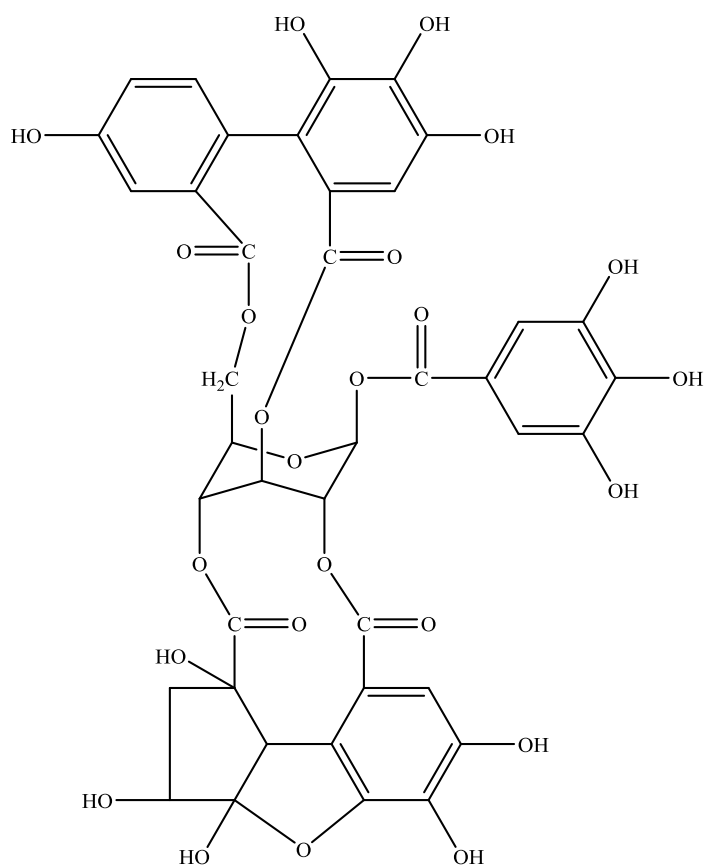


Figure 2.6 (c). Tannins isolated from *Acalypha* sp. - Phyllanthusiin C

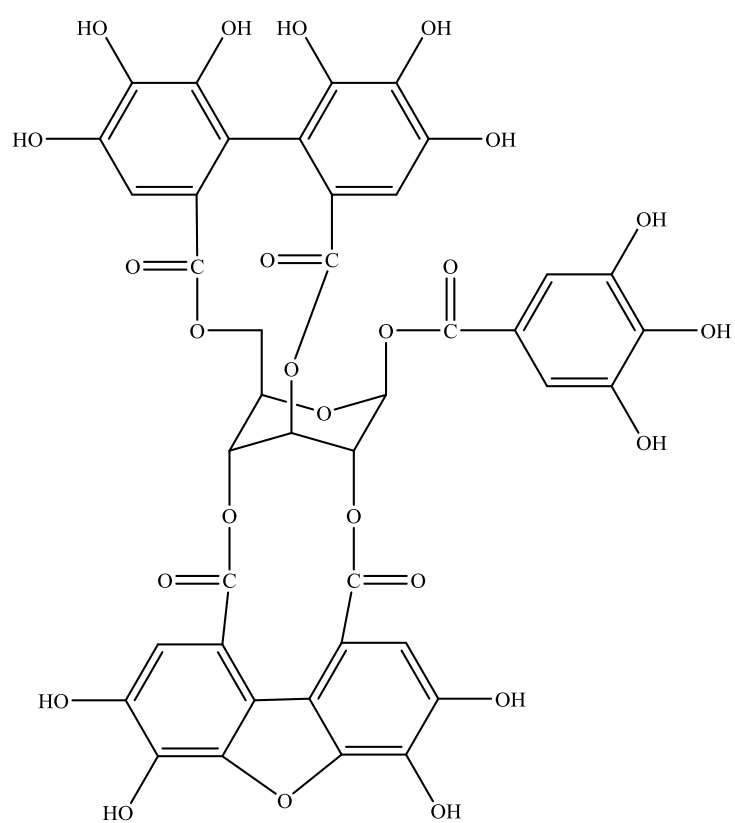


Figure 2.6 (d). Tannins isolated from *Acalypha* sp. - Mallotusin

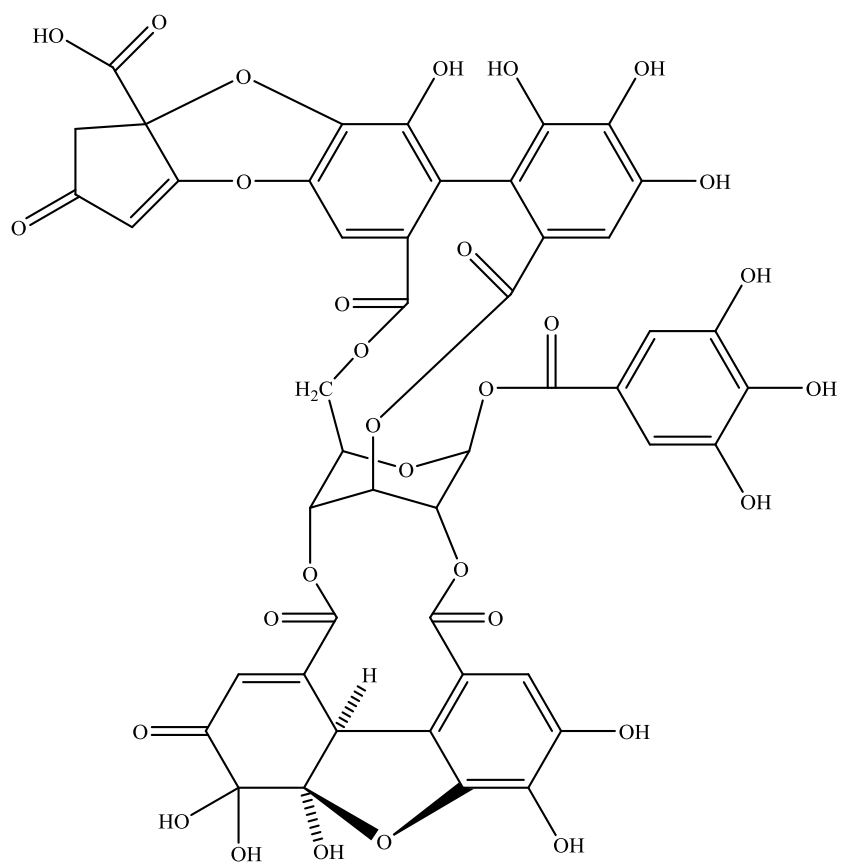


Figure 2.6 (e). Tannins isolated from *Acalypha* sp. - Repandinin A

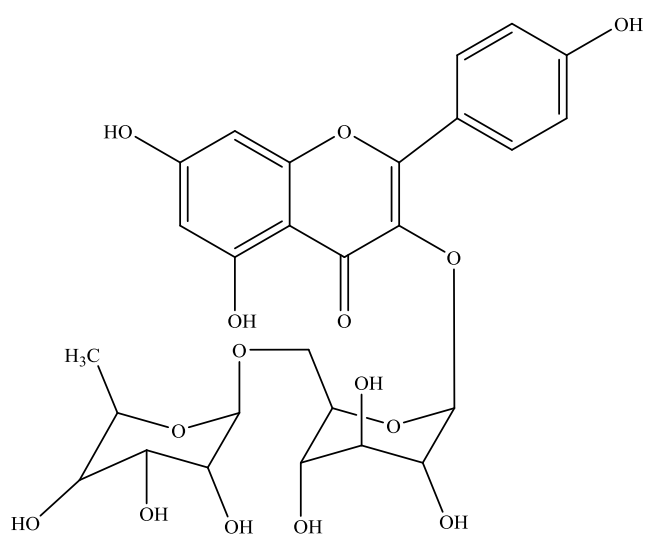


Figure 2.7 (a). Flavonoids, glycosides and terpenes from *Acalypha* sp. -
Kaempferol-3-O-rutinoside

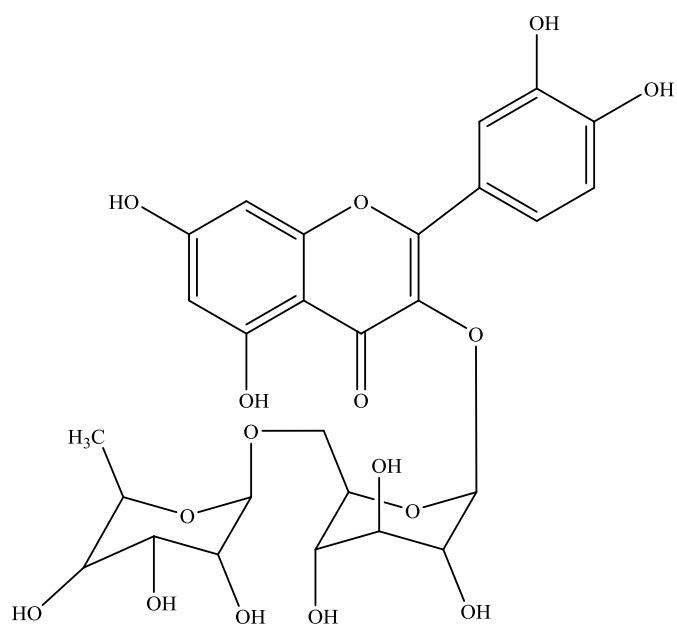


Figure 2.7 (b). Flavonoids, glycosides and terpenes from *Acalypha* sp. -
Quercetin-3-O-rutinoside

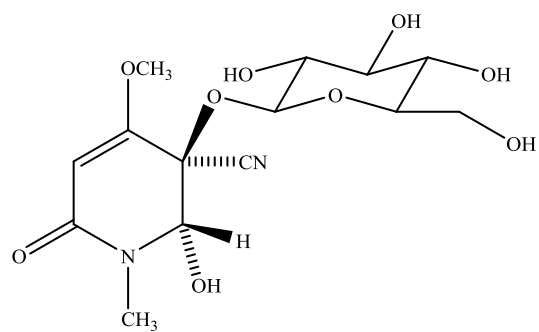


Figure 2.7 (c). Flavonoids, glycosides and terpenes from *Acalypha* sp. -
Acalyphin

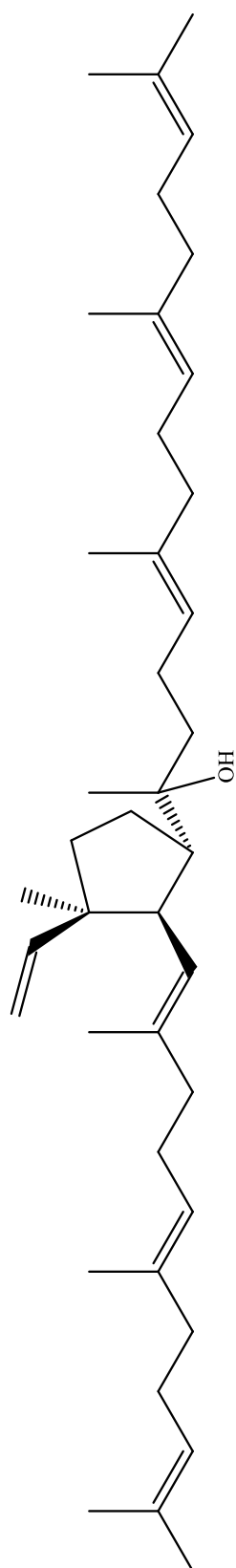


Figure 2.7 (d). Flavonoids, glycosides and terpenes from *Acalypha* sp. - Acalyphaser A

Acalyphin, a cyanogenic glucoside, was isolated by Nahrstedt et al. (1982) from *A. indica* L., and 4 flavonoids were identified from the same plant in 2006 (Nahrstedt et al., 2006). The tetraterpene, Acalyphaser A, was isolated from Malaysian *A. siamensis* Oliv. ex Gage. (Kambara et al., 2006). Tannins (geraniin, corilagin and gallic acid) were elucidated from *A wilkesiana* Müll. Arg. and are claimed to be responsible for its antimicrobial property (Adesina et al., 2000). Besides the isolations of the compounds, there are many researchers that opt to only identify the presence of the phytochemical constituents by executing phytochemical screenings on the extracts. The phytochemical screenings also coincides with the isolation, which leads to terpenoids, flavonoids and tannins being the major compounds in *Acalypha* species (Mothana et al., 2008).

2.5 *Acalypha wilkesiana* var. *macafeana* hort.

The name *Acalypha wilkesiana* originated from Greek, *a*, which means “without” and *kalyphos*, which means “cover”. The plant was named after Admiral Charles Wilkes, a 19th century American naval officer and explorer in the South Pacific (Wiart 2006). Common names are beef steak plant, Jacob’s coat, copper plant or fire dragon. Locally in Malaysia, it is called “*Akalifa*” (Ong 2006).

2.5.1 Botanical Features

A. wilkesiana var. *macafeana* hort. (Figure 2.8) is a tropical bush with variegated copper-red leaves which are ovate to elliptic shaped. Its leaves are 10 -20 centimeters long and arranged in simple and spiral arrangement. This plant can grow up to 4 meters high and is cultivated in Malaysia and all over the Asia-Pacific region as an ornamental plant. The inflorescences are spikes bearing male and female flowers at the base. The flowers are apetalous and without nectar disc. Its flowering season is between February to December (Sagun et al., 2010).

2.5.2 Pharmacological Properties

According to Ong (2006), the leaves are traditionally used in Malaysia in treatments for inflammation, headache, fever and flu, whereas its roots are boiled to wash haemorrhoids. A beverage consisting of about 60 g of the leaves are boiled in goats milk is drunk to lower blood temperature, treat fever, relieve cough and heal pimples. It is also said that 10 g of the leaves boiled with sugar is used to treat thrombocytopenic purpura and allergic purpura which are skin disorders caused by bleeding underneath the skin (Wiart 2006). It is also used in other tropical countries to traditionally treat other diseases such as *Pityriasis versicolor*, tinea and other dermatological disorders, malaria and breast tumors (Büssing et al., 1999; Oyelami et al., 2003; Akinyemi et al., 2005).

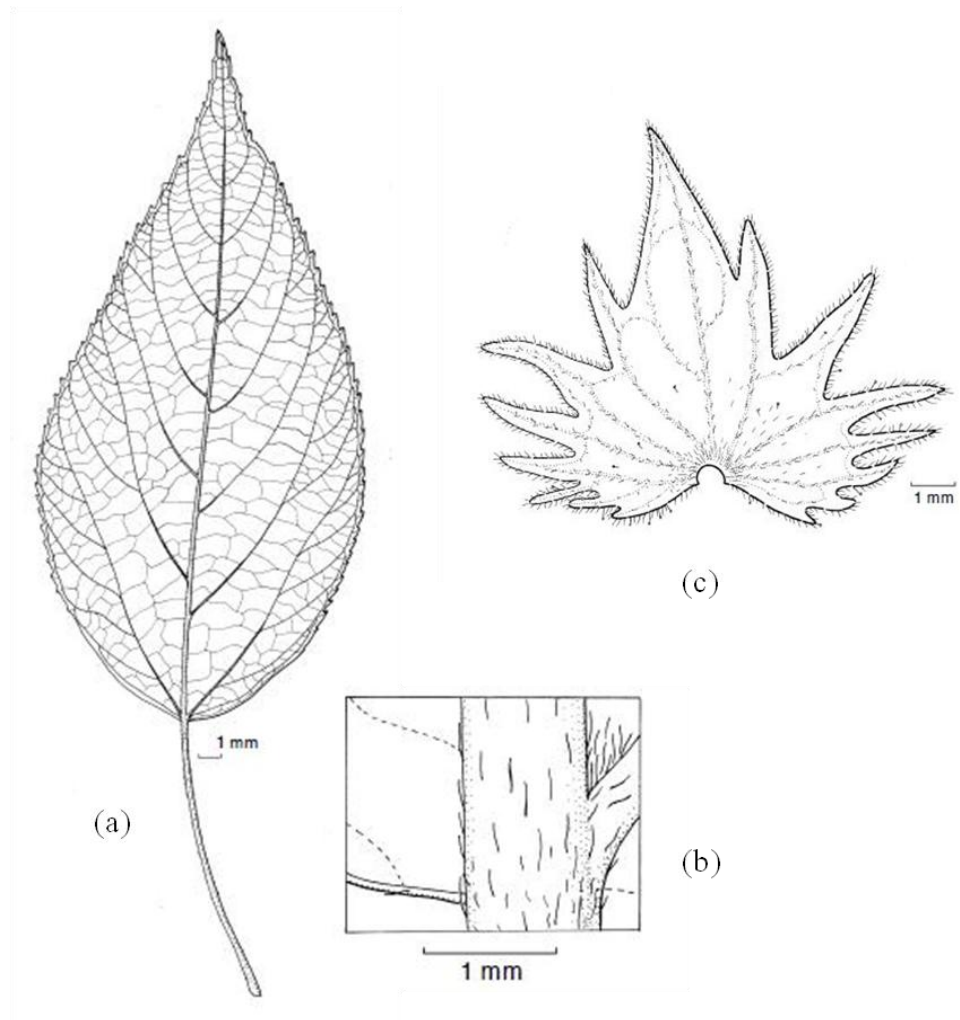


Figure 2.8. Morphology of *A. wilkesiana* a) Leaf morphology b) Details of the leaf morphology showing the indumentum c) Pistillate bract morphology (Sagun et al., 2010).

In West Africa, the plant is used in the form of decoction, infusion or tincture. The leaves are rubbed in between palms and the juice is smeared on the affected part of a patient for treatment of *Pityriasis versicolor* and other skin infections. Grinded leaves may be used as soap to wash affected parts of the skin (Adesina et al., 1980).

2.5.3 Research Commenced on *Acalypha wilkesiana*

To date, the pharmacological properties of *A. wilkesiana* var. *macafeana* hort. are unknown, however, some evidence have been produced on *A. wilkesiana* Müll Arg. In West Africa, research on *A. wilkesiana* Müll Arg. started as early as 1980 with Adesina and colleagues from University of Ile-Ife, Nigeria with the phytochemical and biological examination of the leaves, which observed a broad-spectrum inhibition of Gram-positive and Gram-negative organisms with the well diffusion method.

Indeed, water and ethanol extracts of this plant showed antimicrobial properties with standard and local strains of Gram-positive bacteria *Staphylococcus aureus* and fungi *Trichophyton rubrum*, *Trichopyhton mentagrophytes*, *Candida albicans*



Figure 2.9 (a) *Acalypha wilkesiana* var. *macafeana* hort. shrub

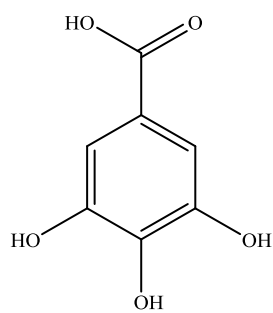


Figure 2.9 (b). *Acalypha wilkesiana* var. *macafeana* hort. leaf morphology.

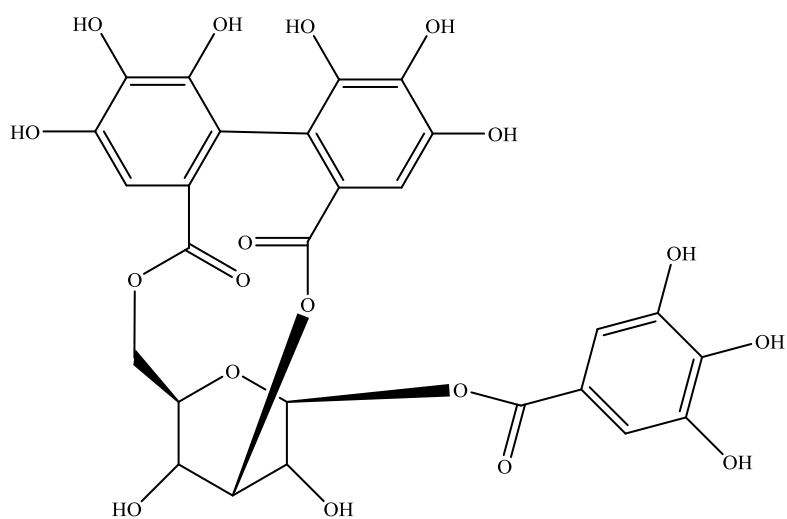
and *Aspergillus flavus* further proving the antiseptic property of this plant (Alade & Irobi 1993). A study *in vitro* on Methicillin Resistant *Staphylococcus aureus* (MRSA) reported good antibacterial activity against MRSA (Akinyemi et al., 2005).

2.5.4 Phytochemical Studies and Isolated Constituents from *Acalypha wilkesiana*

Adesina et al. (2000) found 3 tannins in the leaves of *A. wilkesiana* which are gallic acid, corilagin and geraniin (Figure 2.10), all are said to be responsible for the antimicrobial properties of this plant. Phytochemical screening shows abundance of tannins, saponins and alkaloids in *A. wilkesiana* Müll. Arg and absence of anthraquinones (Soladoye et al., 2008). A comparative phytochemical study on 2 varieties of *A. wilkesiana* Mull Arg. by Oladunmoye et al. (2006) showed presence of tannins, saponin, anthraquinone and cardiac glycoside in both varieties studied. Other screenings also showed similar results of compounds detected (Akinyemi et al., 2005).



(a)



(b)

Figure 2.10 (a). Tannins identified from *A. wilkesiana* Müll Arg. (a) Gallic acid, (b) corilagin

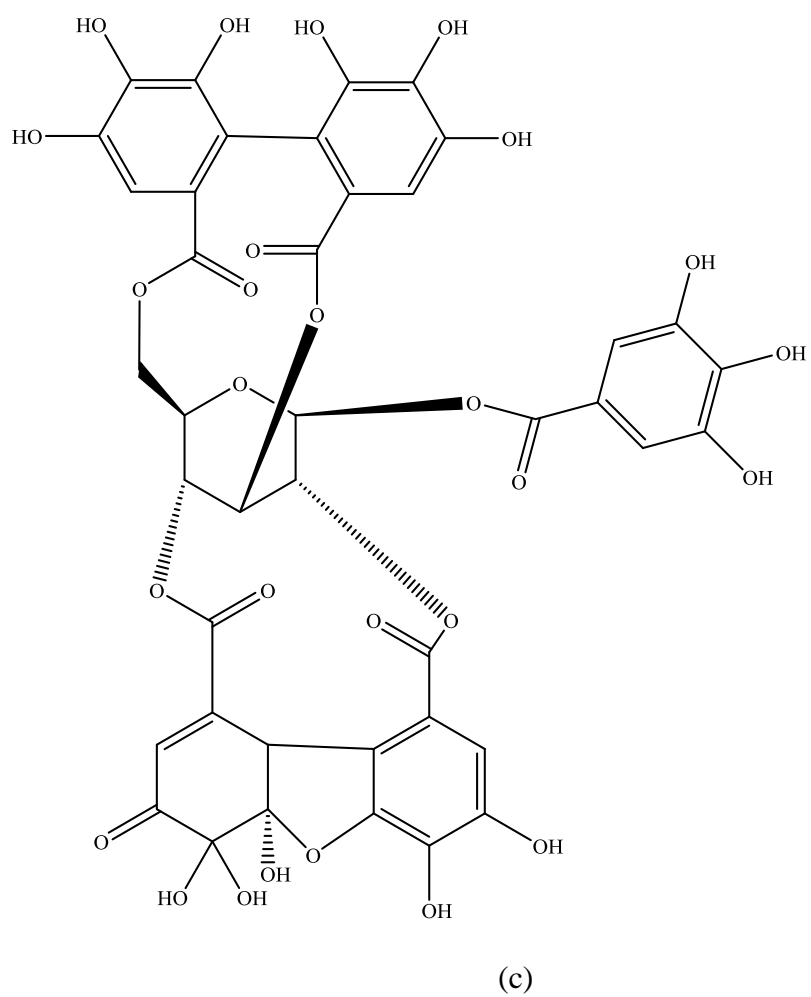


Figure 2.10 (b). Tannins identified from *A. wilkesiana* Müll Arg. (c) Geraniin

2.6 Antibacterial Agents from Flowering Plants

2.6.1 Infectious Diseases

The World Health Organization (WHO) defined infectious diseases as diseases caused by pathogenic microorganisms such as bacteria, fungi, parasites or viruses, which can be spread to another individual either directly or indirectly (Finlay 2010). The latest statistic report from WHO shows 26.8 % of deaths are due to infectious diseases and infections in South East Asia region. Infectious diseases are the second leading cause of death worldwide, and bacteria which are increasingly resistant to antibiotics are well represented among the killers (Finlay 2010).

The European Centre for Disease Prevention and Control (ECDC) and the Centre for Disease Control and Prevention (CDC), focus discussions to infections by Gram-positive *Staphylococcus aureus*, *Enterococcus* sp., *Enterobacteriaceae* (other than *Shigella* and *Salmonella*), and Gram-negative *Pseudomonas aeruginosa* and *Acinetobacter* sp. due to their significant epidemiologies and emerging resistances within the healthcare system (Magiorakos et al., 2012). These pathogenic bacterium evolves to gain resistance against current antibiotics. According to Asian Network for Surveillance of Resistant Pathogens (ANSORP), MRSA infections accounts for 25.5 % of community-associated infections and 67.4 % of healthcare-

associated infections in Asian countries (Song et al., 2011). In Malaysia, the cause of 10 hospital admissions per 1000 admissions is due to Methicillin resistant *Staphylococcus aureus* (MRSA) infections (Al-Talib et al., 2010).

Resistance in bacteria has made the search for new antibiotics more needed than ever (Gibbons 2008). Infections caused by resistant strain bacteria represent a major public health burden in terms of morbidity, mortality, increased expenditure on patient management and infection control measures (Woodford & Livermore 2009). Bacteria are capable of resisting the action of antibiotics via activation of latent mobile genetic elements, mutagenesis of its own DNA and physical exchange of genetic material with another organism (Dwyer et al., 2009). Worldwide emergence of resistant bacteria has been associated with misuse and increasing use of antibiotics in the healthcare settings and the lack of specific drugs for treating patients (Buffet-Bataillon et al., 2012).

2.6.2 Infections Due to *Staphylococcus aureus*

Staphylococcus aureus, a Gram-positive cocci named from Greek with the meaning ‘golden grape-cluster berry’, is a bacterium which causes a multitude of diseases either due to direct infection or the productions of toxins (Archer 1998). *Staphylococcus aureus* produces toxins which are known as one of the virulence factors that contributes to its ability to cause infections which are

categorized to groups of pyrogenic toxin superantigens (PTSAGs), exfoliative toxins (ETs), leukocidins and other toxins (Verkaik et al., 2010). It is frequently found as part of the normal skin flora on the skin and nasal passages. *Staphylococcus aureus* has been recognized as a major cause of wound excretions and a major human pathogen.

Staphylococcus aureus has a diverse arsenal of components and products that contribute to the pathogenesis of infection, either they act together or alone. Generally, the pathogenesis of infections of *Staphylococcus aureus* has been reviewed by Archer (1998) which comprises of five stages which are:

- 1) Colonization
- 2) Local infection
- 3) Systemic dissemination and/or sepsis
- 4) Metastatic infection
- 5) Toxinosis.

Healthy individuals are colonized by *Staphylococcus aureus* usually in the anterior nares, vaginal and perianal area which are carried without any symptoms for weeks or months (Archer 1998). Local abscess results when the organism is inoculated into the skin from the site of carriage. The infection can either be local or carried into the blood. Once in the blood, the bacteria will spread widely to the whole body and septic shock might occur. Dissemination

of the organism, without specific therapy, will result to a number of infections i.e., endocarditis, septic arthritis or epidural abscess. Finally, if the organism does not invade the bloodstream, local or systemic effects of specific toxins will occur such as toxic shock syndrome and food borne gastroenteritis (Archer 1998).

Occurrence of *Staphylococcus aureus* infection in both hospital and community acquired infections increase steadily as treatments have become more difficult due to emergence of resistant strains (Voss & Doebbeling 1995). In Malaysia, occurrence of *Staphylococcus aureus* infections follows the same trend, as it is the second most common bacterium isolated from blood (Ministry of Health 2008). Among the *Staphylococcus aureus* infections, 26 % of the isolates from clinical specimens in 2008 were of Methicillin-resistant strains (MRSA) (Ahmad et al., 2010). The first community acquired Methicillin resistant *Staphylococcus aureus* (MRSA) case in Malaysia was reported by Shamsudin et al. (2008).

2.6.2.1 Staphylococcal Food Poisoning

Staphylococcus aureus colonization of food may cause gastroenteritis with emesis with or without diarrhea. This condition is called staphylococcal food poisoning and results from ingestion of one or more staphylococcal enterotoxins (Dinges et al., 2000). Staphylococcal food poisoning is a common

cause of food poisoning in the world and the condition usually self resolves within 24 to 48 hours of onset.

2.6.2.2 Skin and Soft-tissue Infections

- Impetigo

Impetigo is a contagious skin infection caused by *Staphylococcus* sp. or *Streptococcus* sp. It is more common in children than in adults. There are two forms of impetigo; non-bullous impetigo and bullous impetigo. The non-bullous impetigo is more common and can be caused by either bacteria. Small red papules are observed and will eventually evolve to small blisters and finally scab over with a characteristic honey coloured crust. In bullous impetigo, which is only caused by *Staphylococcus* sp., the top skin layer (epidermis) and the lower skin layer (dermis) separates due to a toxin that is produced by *Staphylococcus*, thus leading to the formation of a blister (termed *bulla* in medicine). These blisters are fragile and contains yellow liquid often breaks and leaves red and raw skin. Finally a dark crust will develop (Cole & Gazewood 2007).

Mild impetigo can be treated with an antibiotic ointment such as mupirocin. More severe impetigo would require oral antibiotics. Common oral antibiotics prescribed are penicillins (such as Augmentin) and cephalosporins (such as Cephalexin). If treatment with these antibiotics is not effective, an MRSA

infection would be suspected and culture tests would be done. MRSA infections would need antibiotics such as sulfamethaxazole.

- Scalded Skin Syndrome

Scalded skin syndrome is an illness characterized by red blistering of the skin that is similar with a burn or a scald. Scalded skin syndrome occurs mostly in children younger than 5 years old. Antibodies gained against staphylococcal exotoxins are usually acquired during childhood, hence less common in older children and adults. The illness is caused by the release of two exotoxins which are epidermolytic toxin A and B from *Staphylococcus aureus*. The epidermolytic toxins are very tissue and species specific, attacking only certain keratinizing epithelia of mice, hamsters, monkeys and human (Elias et al., 1977).

The epidermolytic toxins released by *Staphylococcus aureus* causes cleavage of the middle layer of the epidermis and ultimately, slippage of the epithelium layer upon gentle pressure (Gemmell 1995). Scalded skin syndrome initiates from a localized staphylococcal infection accompanied by fever, irritability and widespread redness of the skin. Within 24 to 48 hours after onset, fluid filled blister will form and will rupture and cause the skin to look like a burn.

Treatment of scalded skin syndrome usually requires hospitalization with intravenous antibiotics generally necessary. A penicillinase resistant anti-staphylococcal antibiotic such as flucloxacillin can be used. Recovery is usually complete within one week after treatment with the right antibiotic. If the illness is left untreated or treatment is unsuccessful, severe infection will develop such as sepsis, cellulitis and pneumonia and eventually mortality.

- Acne

Staphylococcal acne is medically known as staphylococcal folliculitis. It is an infection of the hair follicle caused by *Staphylococcus aureus* which are localized on an individual, and infects the surface of the skin when there is a break in the skin that occur from shaving, skin friction or rubbing from clothes. These follicular lesions frequently coexisted at the sweat pores (O'Brien 1952). This condition can be treated with topical application of fusidic acid and oral intake of penicillin (Smith 1978).

2.6.2.3 Bacteremia and Pneumonia

Pneumonia caused by *Staphylococcus aureus* occurs in about 1 to 10 % of community-acquired pneumonia (Gonzalez et al., 1999). Diagnosis of pneumonia caused by *Staphylococcus aureus* is made when there are existing symptoms of infection of the lower respiratory tract such as cough, purulent expectorant and chest pain, coinciding with the isolation of *Staphylococcus*

aureus. Pneumonia caused by MRSA is the second most frequent infection after surgical wound infection and is a major factor associated to mortality (Gonzalez et al., 1999). Bacteremia is defined when one or more hemocultures are identified positive for this bacterium, accompanied by symptoms of clinical sepsis such as chills, fever, skin rash, a drop in blood pressure and very fast breathing.

2.6.2.4 Toxic Shock Syndrome

This illness was brought to attention to the medical world in 1978 by Todd et al. who identified toxic shock syndrome as a major systemic illness associated with noninvasive *Staphylococcus aureus* infections in children (Dinges et al., 2000). The toxic shock syndrome is an acute and fatal illness that is characterized by high fever, diffuse erythematous rash, desquamation of the skin, hypotension, and involvement of three or more organ systems (Lowy 1998). In the early 1980's, an epidemic of the toxic shock syndrome occurred among young women in the United States which were later identified as caused by the use of tampons during menstruation (Lowy 1998).

2.6.3 Drugs Available for Treatment of Bacterial Infections

The discovery of penicillin in 1928 by Fleming led to the discovery of many more antibiotics that either successfully make through clinical trials and commercialized or were unsuccessful. The discovery of the first three

antimicrobials i.e., Salvarsan, Prontosil and penicillin was exemplary in the set up for drug discovery (Aminov 2010). Between 1950s and 1970s, were the golden era of discovery of antibiotics, with no new groups of compounds discovered since then (Aminov 2010). These antimicrobials were then classified to different groups of antibiotics following their target and mechanism of action and its chemical structures.

Currently, antimicrobial classes used for infectious diseases include aminoglycosides, carbapenems, cephalosporins, fluoroquinolones, penicillins and vancomycin (Peterson 2005). Coates et al. (2011) listed out β -lactams, cephalosporins, carbapenems, aminoglycosides, tetracyclines, macrolides, glycopeptides, sulphonamides and quinolones as main classes of antibiotics, in which some are commercialized and some are withdrawn.

2.6.3.1 Treatment for *Staphylococcus aureus* Infections

Penicillin still remains the drug of choice for isolates that is sensitive to it. For β -lactamase producing strains, a semisynthetic penicillin (oxacillin) is used. Vancomycin is preferred for methicillin-resistant strains, though for patients that are unable to tolerate vancomycin, they can be treated with fluoroquinolones, clindamycin or minocycline (Lowy 1998). Antimicrobial combinations have been used to increase bactericidal activity or prevent development of resistance. The combination of β -lactams and aminoglycosides

increases bacterial killing in vitro and in animal models of endocarditis of *Staphylococcus aureus* (Miller et al., 1978).

The use of topical agents to eliminate *Staphylococcus aureus* colonization in high risk groups, such as patients undergoing dialysis or surgery, has been shown to reduce the incidence of infections (Yu et al., 1986). Muciprocin which is an anti-staphylococcal topical agent that inhibits RNA and protein synthesis, eliminates nasal colonization and can reduce the incidence of wound infections (Reagen et al., 1991).

2.6.4 Action Mechanism of Antibiotics

Antibiotics can be categorized by their modes and site of action. There are antibiotics which are inhibitors of cell wall synthesis, disruptors of cell membranes, inhibitors of protein synthesis and inhibitors of nucleic acid synthesis as tabulated in Table 2.2.

Table 2.2. Antibiotics categorized by their modes of action.

Mode and site of action	Antibiotic	Origin
Inhibitors of cell wall synthesis	Penicillins (Penicillin G, oxacillin, ampicillin, amoxicillin)	Fungi <i>Penicillium notatum</i> or <i>Penicillium chrysogenum</i>
	Cephalosporins (Cephalexin, cefradroxil)	Fungi <i>Cephalosporium</i> sp.
	Carbapenems (Bacitracin, primaxin, vancomycin)	Bacitracin – <i>Bacillus lincheniformis</i> Vancomycin – <i>Streptomyces orientalis</i>
	Polymixin (Polymixin B)	<i>Bacillus polymyxa</i>
Inhibitors of protein synthesis	Aminoglycosides (Streptomycin, gentamicin, kanamycin)	<i>Streptomyces</i> and <i>micromonospora</i> sp.
	Tetracycline	
	Chloramphenicol	<i>Streptomyces</i> sp.
Inhibitors of nucleic acid	Rifampin	Semi synthetic, originally from <i>Streptomyces mediterranei</i>
	Quinolones	Synthetic

2.6.4.1 Inhibitors of Cell Wall Synthesis

β -lactam antibiotics are a broad class of antibiotics which consists of all antibiotics which contain a β -lactam nucleus in their molecular structure (Figure 2.11). These include penicillin derivatives, cephalosporins, and carbapenems (Holten & Onusko 2000). All these antibiotics target the cell wall. β -lactam antibiotics inhibit the growth of bacteria by inactivating enzymes located in the bacterial cell wall. They act by covalently combining with penicillin binding proteins (PBPs) and inactivating transpeptidase and carboxypeptidase activities that are responsible for catalyzing the final transpeptidation step of bacterial cell wall biosynthesis (Waxman & Strominger 1983).

The effectiveness of β -lactam antibiotics relies on their ability to reach the penicillin binding protein (PBP) intact and ability to bind to PBP. Hence, resistance to β -lactam antibiotics occurs by four primary mechanisms (Drawz & Bonomo 2010):

- 1) Production of β -lactamase enzyme which will hydrolyze the β -lactam ring of the antibiotic, rendering the antibiotic ineffective
- 2) Low affinity of the active site of PBPs for β -lactam antibiotics through natural transformation and recombination
- 3) Decreased expression of outer membrane proteins as β -lactams must diffuse through or traverse porin channels in the outer membrane of

Gram-negative cell walls in order to access the penicillin binding protein (PBPs) on the inner plasma membrane. This mechanism of resistance has been identified for carbapenem antibiotics.

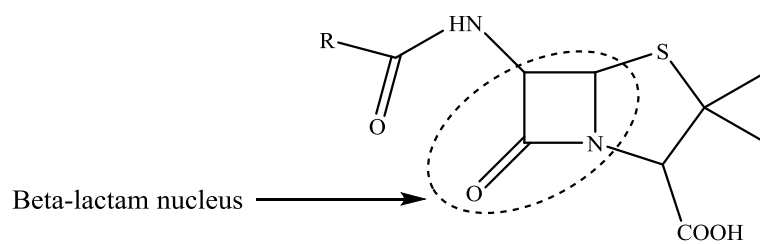
- 4) Upregulation of the efflux pump which are capable of exporting substrates from the periplasm to the surrounding environment can contribute to decreased susceptibility to penicillins and cephalosporin.

- Penicillins

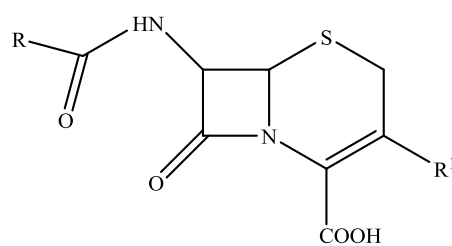
Penicillin is a group of antibiotics derived from *Penicillium* fungi (Black 2002). Some commercially used derivatives of penicillin includes penicillin V, amoxicillin, ampicillin, oxacillin and carbenicillin. Penicillin G is the most used natural penicillin that is used in treating infections caused by streptococci, meningococci, pneumococci and clostridia (Black 2002). Other penicillins such as ampicillin, oxacillin and amoxicillin are semi-synthetic. In addition to their use as treatments for infections, penicillins are also used to prevent infection. For example, patients are given penicillin before dental surgery to prevent infections.

- Cephalosporins

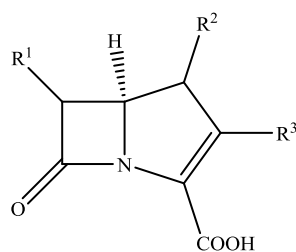
Cephalosporin antibiotics are the second major groups of β -lactam antibiotics after penicillins. Classification of cephalosporins is by generation, as it reflects



(a)



(b)



(c)

Figure 2.11. β -lactam pharmacophore in the molecular structure of β -lactam antibiotics (a) Penicillin, (b) Cephalosporin and (c) Carbapenem. (R, R₁, R₂ and R₃: different functional groups)

their antibacterial activity. First generation cephalosporins have good activity against Gram-positive cocci. Second generation cephalosporins have enhanced activity against Gram-negative bacteria. A further increase in potency against Gram-negative bacteria, including against *Enterobacteriaceae* such as *Pseudomonas* sp, *Klebsiella* sp. and *Shigella* sp, is a characteristic of the third generation cephalosporins, though these compounds are less active against Gram-positive cocci compared to the first generation agents. The fourth generation cephalosporins are active against both Gram-positive and Gram-negative bacteria and are more stable against β -lactamases (Mason & Kietzmann 1999). Cephalosporins includes first generation cefadroxil and cephadrine, second generation cefaclor and cefprozil and third generation cefixime and cefdinir (Holten & Onusko 2000). Fourth generation cefepime is also reported (Mason & Kietzmann 1999).

Cephalosporins are bactericidal and acts by inhibiting cell wall synthesis. Bacterial resistance is known by the production of enzymes which hydrolyze the β -lactam ring. Currently, cephalosporins are used for urinary and respiratory tract infections, otitis media, meningitis and in skin and soft tissue infections (Mason & Kietzmann 1999).

- Carbapenems

Carbapenems are a new β -lactam bactericidal antibiotic which is characterized with the β -lactam ring with a carbon instead of a sulfone in the moiety (Figure 2.11). The first carbapenem to be used was the antibiotic imipenem, which was coadministered with cilastatin, a compound that prevents the degradation of the compound in the kidneys (Bonfiglio et al., 2002). Other carbapenems in use are meropenem and doripenem. The mechanism of action of carbapenems are same with other β -lactams, by inhibiting the cell wall synthesis via inactivation of transpeptidase and inhibition of the formation of peptide cross linking bonds, thus breaking the cell wall integrity.

2.6.4.2 Disruption of Bacterial Membrane

- Polymixin

Polymixins are polypeptide antibiotics which are effective especially against Gram-negative bacteria, as they act upon the cell membrane. Polymixins act as detergent and distorts the bacterial cell membrane, by binding to phospholipids. Distortion of the cell membrane results in leakage of the cell contents and thus fatal to the bacteria (Kioke et al., 1969). Gram-negative bacteria membranes are rich in phospholipids.

2.6.4.3 Inhibitors of Protein Synthesis

- Aminoglycosides

Aminoglycosides refers to a family of molecules containing amino-modified sugars linked by glycosidic bonds, and functions as antibiotics (Figure 2.12). Currently available aminoglycoside antibiotics are streptomycin, gentamicin, kanamycin and neomycin. Aminoglycosides are used in the treatment of severe infections of the abdomen and urinary tract, bacteremia, and endocarditis (Jana & Deb 2006). It has the advantage to elicit broad antimicrobial spectrum, rapid bactericidal action and ability to act synergistically with other drugs (Jana & Deb 2006). Due to their structure, these antibiotics are considered polycationic, which enable them to show binding affinity for negatively charged residues in the outer membrane and in nucleic acids such as the 16S rRNA of the 30S ribosome.

Aminoglycosides are bactericidal by inhibiting protein synthesis as they bind to the 16S rRNA, a component of the 30S small subunit of ribosome, and disrupts the integrity of bacterial cell membrane. Resistance with this type of antibiotics occur with mechanisms including (Shakil et al., 2008):

- 1) Deactivation of aminoglycosides by N-acetylation, adenylation or O-phosphorylation

- 2) Reduction of intracellular concentration of aminoglycosides by changes in outer membrane permeability, decreased inner membrane transport, active efflux and drug trapping
- 3) Alteration of the 30S ribosomal subunit target by mutation
- 4) Methylation of the aminoglycosides binding site.

- Tetracyclines

Tetracyclines are a group of broad-spectrum antibiotics which are named for their four ('tetra') hydrocarbon rings (naphthacene ring). Tetracycline derivatives are different with substitution at position 5, 6 and 7 which is the conserved region (Figure 2.13). The pharmacophore lays with position 2, 3, 4, 10, 11, and 1 where modifications are not tolerated without loss of activity. Tetracycline penetrates bacterial cells by passive diffusion and inhibits bacterial growth by interfering with protein synthesis. This happens as tetracycline binds to the 30S subunit of the ribosome during protein synthesis, and inhibits binding of the t-RNA to the matching codons. As a result, protein synthesis does not occur (Chopra & Roberts 2001).

There are two groups of tetracycline distinguished by its different mechanism of action, which are the typical tetracycline (bacteriostatic, Figure 2.14a) and tetracycline derivatives which are bactericidal (Schnappinger & Hillen 1996).

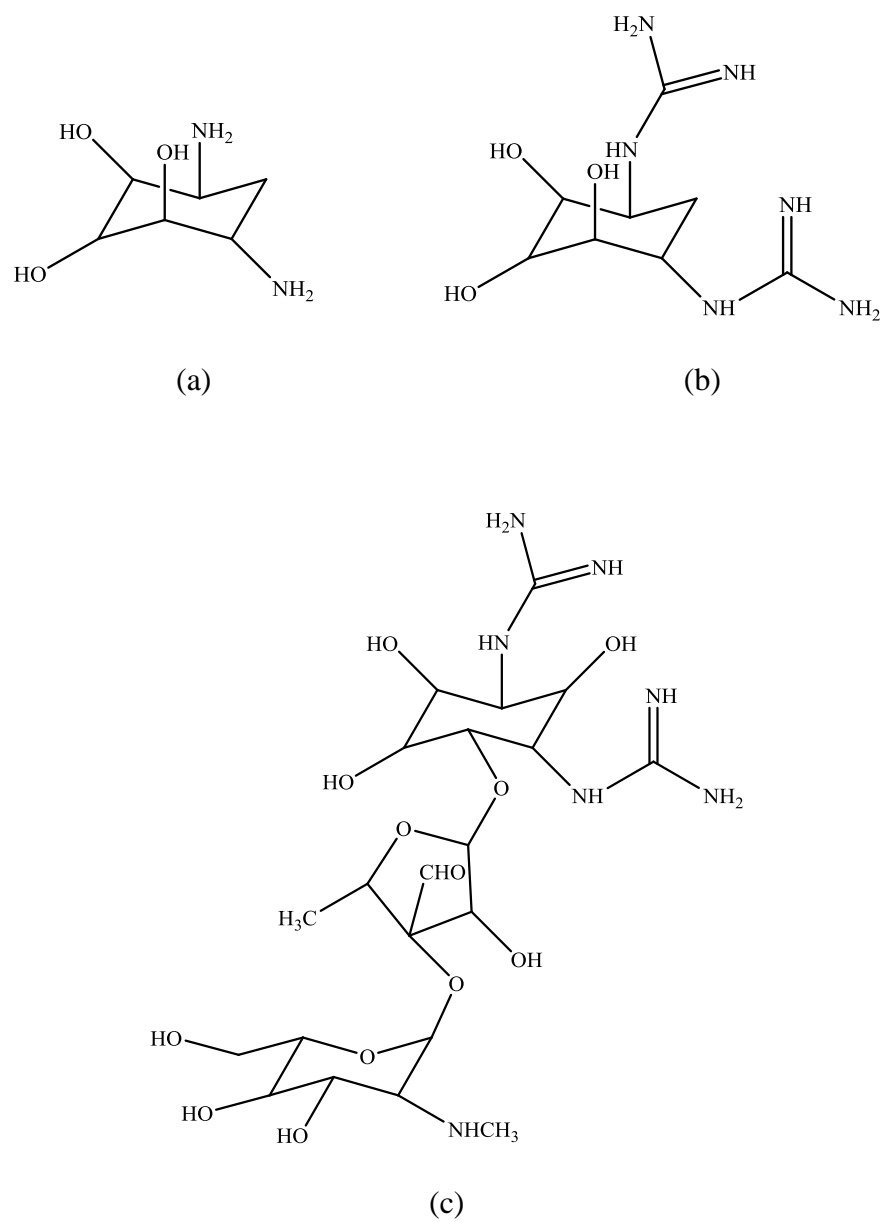
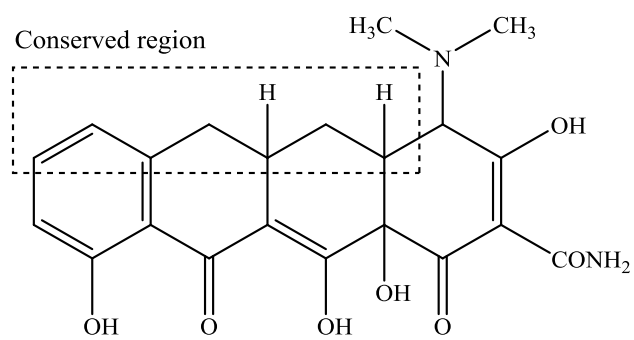


Figure 2.12. Backbone structures of aminoglycosides (a) 2-deoxystreptamine (b) streptidine and (c) streptomycin.

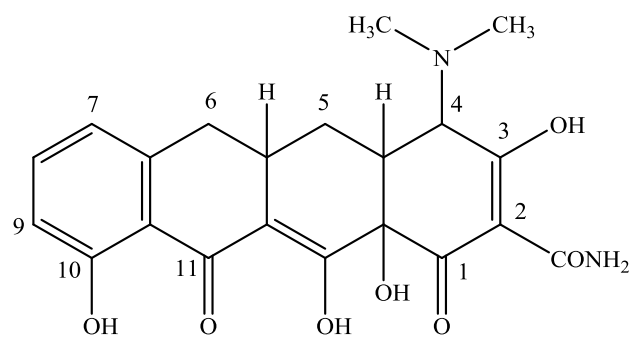
The bacteriostatic mechanism of typical tetracyclines such as tetracycline, doxycycline and minocycline, are associated with reversible inhibition of protein synthesis by reversible binding of the tetracycline to the ribosome, thus distorting the alignment of the anticodons of the t-RNA to the codons of the mRNA.

Chelocardin, anhydrotetracycline (hydrochloride) and 6-thiatetracycline (Figure 2.14b) are tetracycline agents that are bactericidal due to their ability to cause morphological alterations of the bacterial cell and cause cell lysis (Oliva et al., 1992). This is reflected by the release of β -galactosidase from the cytoplasm. These agents may promote cell lysis and death by interfering with electrochemical gradient thus, stimulates autolytic enzyme activity and cellular lysis (Oliva et al., 1992). Although the latter group of tetracyclines are bactericidal, these derivatives are of no therapeutic value as its application leads to severe side effects (Rogalski 1985).

According to Speer et al. (1992), bacteria use three strategies to derive resistance to tetracycline: by limiting access of tetracycline to ribosomes, by altering the ribosome to prevent effective binding of tetracycline, and by producing tetracycline inactivating enzymes. All of these three types of resistance have been found in clinical isolates.

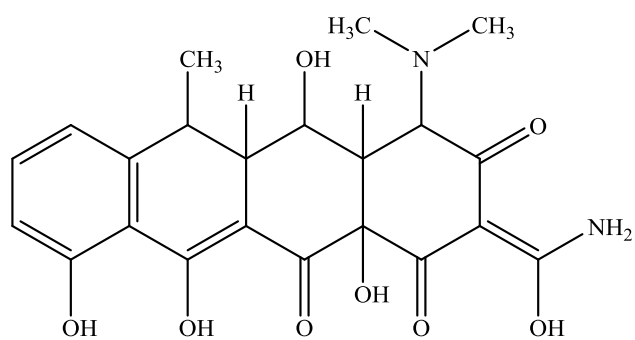


(a)

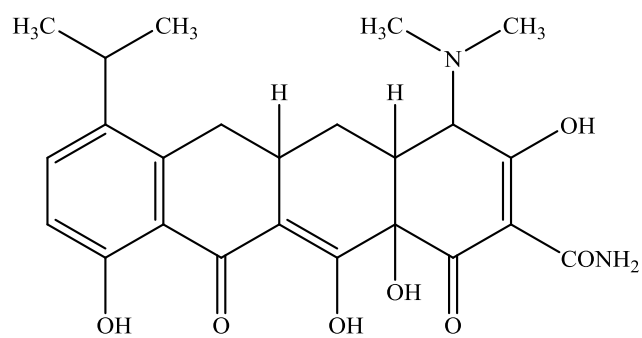


(b)

Figure 2.13. Conserved region and numbering scheme for tetracyclines antibiotics

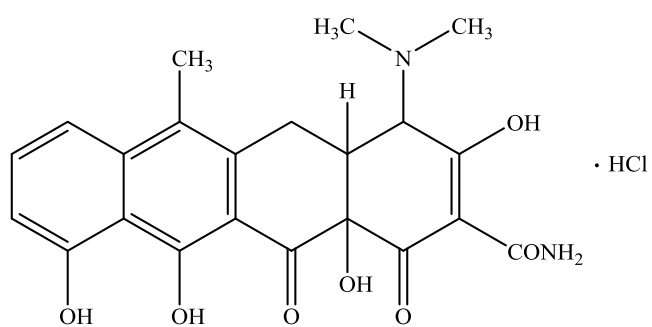


(a)

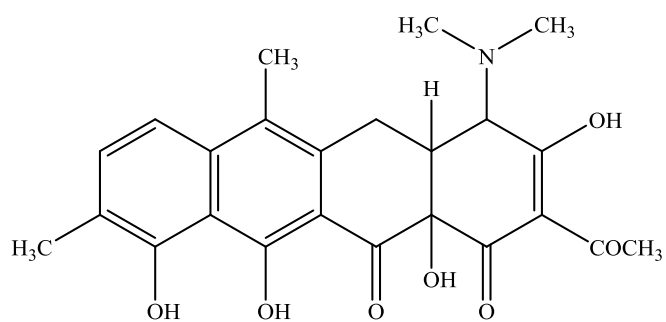


(b)

Figure 2.14a. Typical tetracycline structures which are bacteriostatic. (a) Doxycycline, (b) Minocycline



(a)



(b)

Figure 2.14b. Tetracycline derivatives which are bactericidal. (a) Anhydrotetracycline (hydrochloride), (b) Chelocardin.

- Chloramphenicol

Chloramphenicol is a bacteriostatic antibiotic which originally is obtained from *Streptomyces venezuelae*. Currently this antibiotic is fully synthesized in laboratories. It has a broad spectrum of activity and is usually prescribed for typhoid fever, meningococci infections, *Haemophilus influenza* infections, brain abscess and *Rickettsiae* infections (Black 2002). Although it is an effective broad spectrum antibiotic, it is not the first line antibiotic of use as it is known to cause serious side effects such as elevated chloramphenicol serum concentrations, anemia, thrombocytopenia, reticulocytopenia, and severe metabolic acidosis (Wiest et al., 2012). Currently, chloramphenicol is prescribed for eye infections.

Chloramphenicol is bacteriostatic by inhibition of protein biosynthesis via inhibition of transpeptidation. Chloramphenicol binds to the 50S subunit of the ribosome and blocks the binding of aminoacyl moiety of the tRNA to the mRNA complex. Peptide at the donor site is thus unable to be transferred to the amino acid acceptor and inhibits synthesis (Balbi 2004).

2.6.4.4 Inhibitors of nucleic acid

- Rifamycin

Rifampicin is a semi-synthetic antibiotic from the group rifamycin produced by *Streptomyces mediterranei* which blocks RNA transcription (Black 2002).

Although it is a broad spectrum bactericidal antibiotic, it is only approved for the treatment of tuberculosis and eliminating meningococci from the nasopharynx of carriers as resistance towards rifampin develops rapidly (Black 2002). Treatments of tuberculosis patients with rifampin has to be daily, strictly monitored and cannot be stopped due to the rapid development of resistance towards the drug.

Rifamycin specifically inhibits bacterial RNA polymerase which is the enzyme responsible for DNA transcription, by forming a stable drug-enzyme complex. Mammalian enzymes are not affected by rifamycin. Bacterial resistance is usually caused by mutation which leads to a change in the structure of the RNA polymerase (Wehrli 1983)

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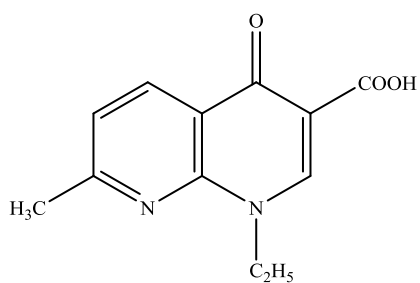
- Quinolones and Fluoroquinolones

Quinolones are a group of synthetic antibiotic which are effective against Gram-positive and Gram-negative bacteria. Fluoroquinolones are a major subset of this antibiotics which have a fluorine atom attached to the ring at C-6 or 7 position (Figure 2.15). The first quinolone was nalidixic acid which was introduced in the 1960's, then subsequently, the second, third and fourth generation of quinolones was introduced. Table 2.3 shows the first, second, third and fourth generation quinolone antibiotics.

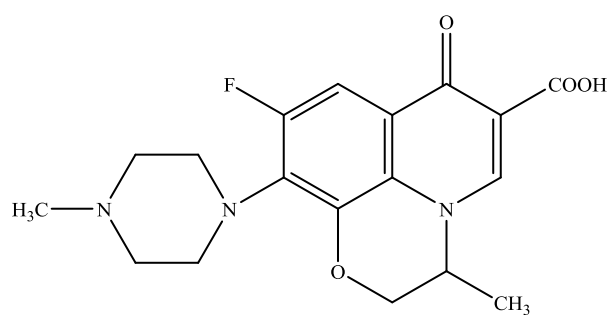
Table 2.3. First, second, third and fourth quinolone antibiotics (Class et al. 2002).

Generation	Antimicrobial activity	Improvements
First	Enterobacteriaceae	Narrow Gram-negative coverage
Nalidixic acid		Oral administration
Cinoxacin		
Second (Class I)	Enterobacteriaceae	Improved Gram-negative coverage
Lomefloxacin		Limited Gram-positive coverage
Norfloxacin		Oral administration
Ecoxacin		
Second (Class II)	Enterobacteriaceae	Broader spectrum of pathogens
Ofloxacin	<i>Pseudomonas aeruginosa</i>	Oral and intravenous administration
Ciprofloxacin	(Ciprofloxacin)	Higher serum, tissue and intracellular drug concentrations
Third	Enterobacteriaceae,	Broader spectrum including Streptococci
Levofloxacin	<i>Pseudomonas aeruginosa</i> ,	Oral and intravenous administration
Sparfloxacin	Streptococci	

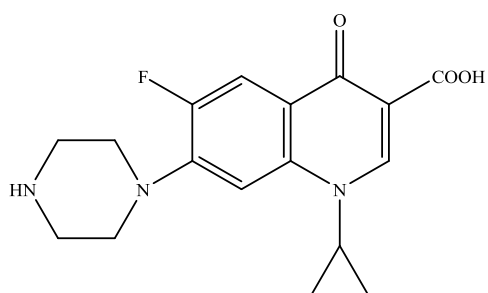
Moxifloxacin		
Trovafloxacin	Enterobacteriaceae, <i>Pseudomonas aeruginosa</i> , MSSA, Streptococci	Improved Gram-positive coverage Oral and intravenous administration



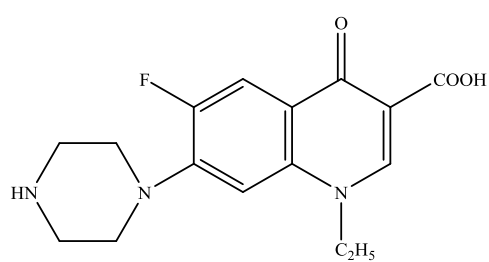
(a)



(b)



(c)



(d)

Figure 2.15. Quinolones and fluoroquinolones antibiotic. (a) Nalidixic acid, (b) Ofloxacin, (c) Ciprofloxacin, (d) Norfloxacin.

Quinolones and fluoroquinolones inhibits bacterial replication by inhibiting DNA replication. DNA replication is essential as it contains the genetic material which enables the bacteria to function. In replication, the double stranded DNA is unwind to a single stranded form which allows synthesis of mRNA. This unwinding of the double stranded DNA is done by a topoisomerase II enzyme which is DNA gyrase. Quinolones and fluoroquinolones bind to a subunit of this enzyme and alters the active binding site of the enzyme, inhibiting it to continue the replication of the DNA (Hooper 1999).

2.6.5 Isolated Antibacterial Agents from Flowering Plants

Approximately 11500 from 30000 antibacterial agents are isolated from flowering plants according to Berdy (2005), demonstrating the importance of flowering plants as a source of antibiotics. Plants have unlimited ability to synthesize metabolites which serve as plant defense against microorganisms (Cowan 1999). Reported to be antibacterial metabolites are phenolics, terpenoids, alkaloids, polypeptide and polyacetylenes (Cowan 1999).

Among the earliest isolation of antibacterial from plants would be the isolation of eugenol from cloves (*Syzygium aromaticum* (L.) Merr. & L.M. Perry) and thymol from thyme (*Thymus mongolicus* (Ronniger) Ronniger) (Hitokoto et

al., 1980). Quinine is another alkaloid that has been identified with antibacterial properties isolated from *Cinchona calisaya* Wedd. (Greenwood 1992). *Cinnamomum osmophloeum* Kaneh. has shown to possess antibacterial property against *Escherichia coli*, *Enterococcus faecalis*, *Staphylococcus aureus* and Methicillin-resistant *Staphylococcus aureus* (MRSA) with cinnamaldehyde being the main antibacterial component.

Recently, antibacterial compounds are being actively isolated from plants using better technologies in elucidation of compounds such as alkaloids, phenolics and terpenes. Quidoline and cryptolepine were identified from GC-MS analysis of an alkaloid extract of *Sida acuta* Burm f. used traditionally in Africa (Karou et al., 2011). A compound oleanolic acid isolated from *Carpobrotus edulis* (L.) N.E. Br. was identified very active against *Enterococcus faecalis* and *Staphylococcus aureus*. It has also shown inhibition of *Mycobacterium tuberculosis* (Martins et al., 2011). Eight compounds are isolated from *Trichodesma indicum* (L.) Lehm. root extract and all eight compounds are antibacterial with a glucoside of a tetracyclic triterpene lactone (lanast-5-en-3 β -D-glucopyranosyl-21 (24)-olide) being the most active compound against *Staphylococcus aureus* at a minimum inhibition concentration value of 2.4 μ g/mL (Periyanayagam et al., 2012).

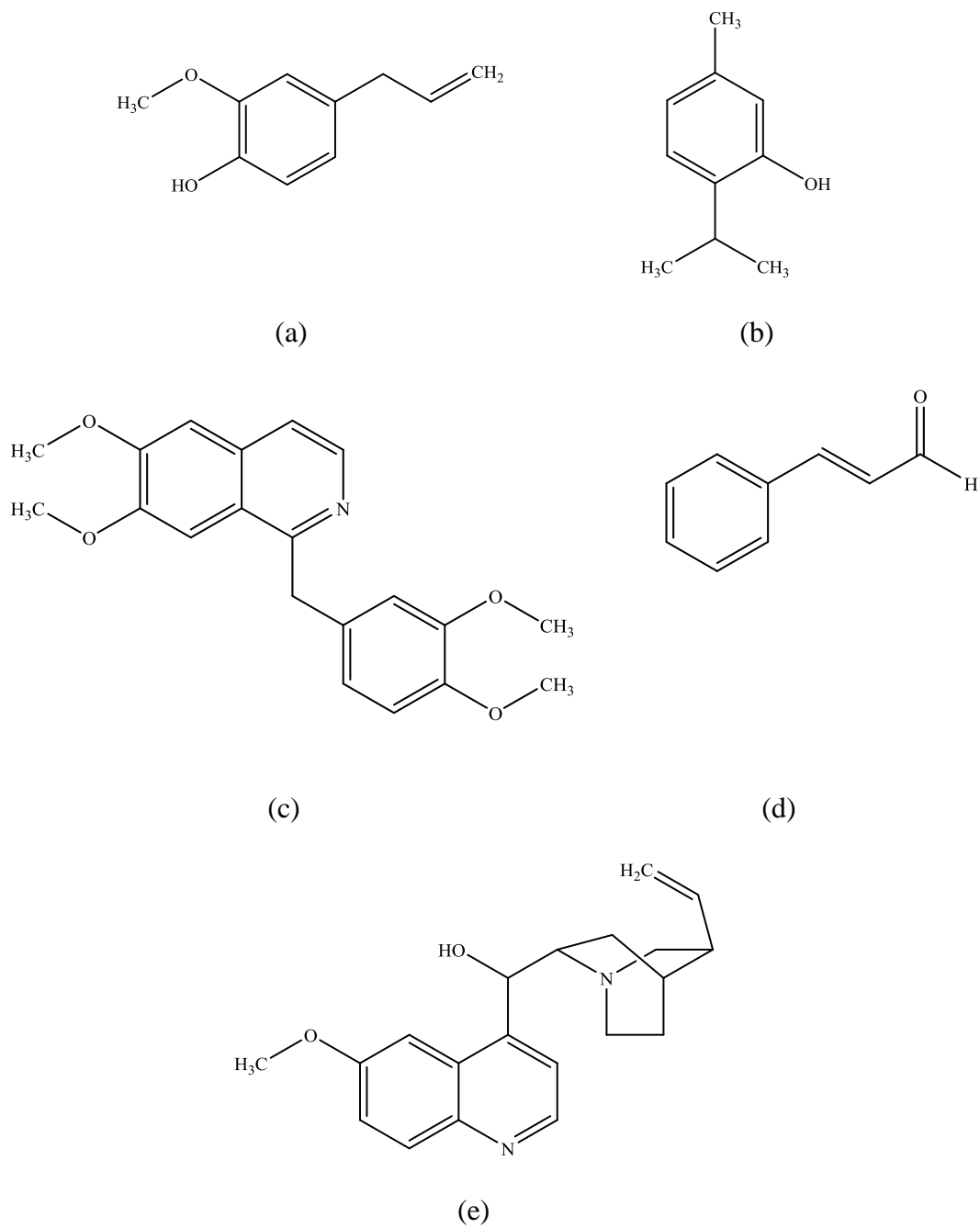
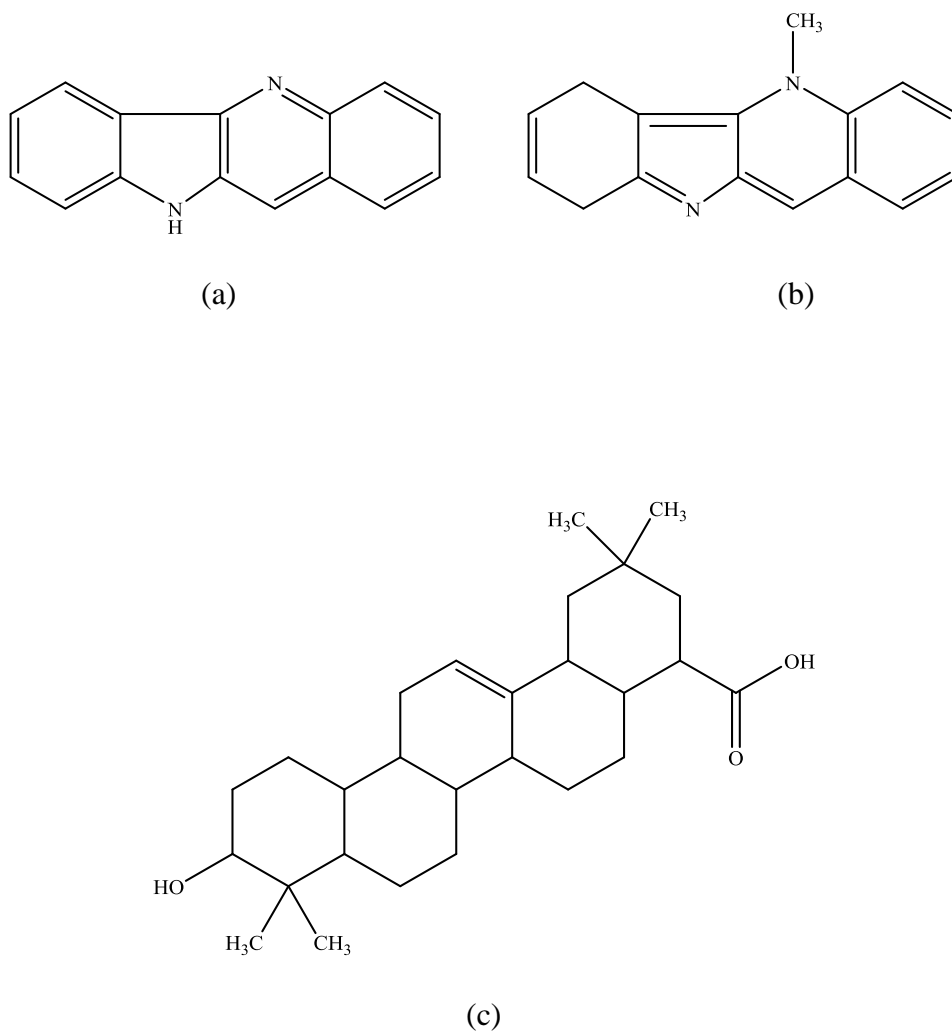
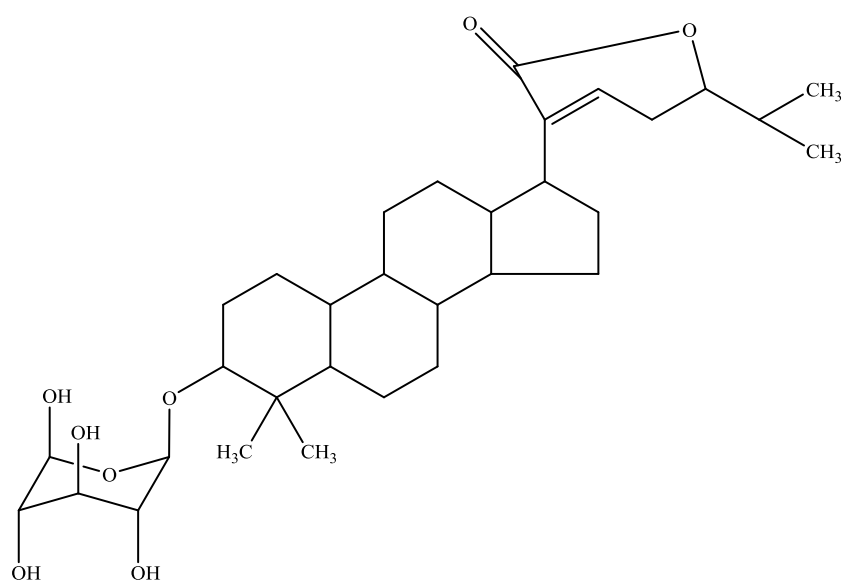


Figure 2.16. Antibacterial agents isolated in the earlier years (a) Eugenol, (b) Thymol, (c) Cinnamaldehyde, (d) Papaverine, (e) Quinine



Figures 2.17 (a). Antibacterial agents isolated from flowering plants (a) Quidoline, (b) Cryptolepine, (c) Oleanol acid.



Figures 2.17 (b). Antibacterial agents isolated from flowering plants - Lanast-5-en-3β-D-glucopyranosyl-21 (24)-olide.

2.6.6 Tests to Assess Antibacterial Properties

Methods to assess antibacterial properties of compounds and medicinal plants are classed into three groups which are diffusion method, dilution method and bioautographic method (Rios et al., 1988).

2.6.6.1 Diffusion Method

In a diffusion method, the sample is brought into contact with the bacteria via a reservoir containing the sample. The reservoirs commonly used are cups, holes in agar or discs. Upon incubation, the diameter of a clear zone around the reservoir indicates inhibition of the bacterium. This method was originally applied to assess effectiveness and selectivity of antibiotics (Rios et al., 1988).

Diffusion methods are the most employed method in antibacterial research due to its advantages. The advantages of diffusion methods are the small size of the sample used in screening and the possibility of testing five or more compounds against a single microorganism. Diffusion methods are well suited for preliminary screening of pure substances and crude extracts. Although it is widely used by many researchers, the disadvantage to these methods is that it is not appropriate to be used with samples that are not soluble in water such as essential oils and non polar extracts. Diffusion methods can be used as a screening tool, but results from it alone can never be used for definitive

activity, thus, it is a common practice to test with more than one method for definite assessment.

2.6.6.2 Dilution Method

Dilution techniques are methods which require a homogenous dispersion of the sample in broth or water. Dilution methods are commonly used to determine the minimum inhibition concentration (MIC) and the minimum bactericidal concentration (MBC) of compounds or extracts. In the dilution method, turbidity is an indication of bacterial density and growth. Thus, clear medium indicates no growth. Inhibitions are indicated to the turbidity of the medium and are measured either visually or by a spectrophotometer (Rios et al., 1988). Dilution method can be done in either liquid or agar form. Dilution in liquid medium is more laborious but is the most precise technique (Rios et al., 1988). Currently, the microbroth dilution method is sought for high throughput screening of extracts and compounds as it only requires a small amount of sample and accommodates more samples to be assessed.

2.6.6.3 Bioautographic Methods

Bioautographic methods are *in situ* method applying the biological effect that is assessed, in this case antibacterial. Bioautographic methods are commonly using paper chromatography or thin layer chromatography, whereby the antibacterial compound is transferred from the chromatographic layer to an

inoculated agar plate. Inhibition zones are visualized by dehydrogenase activity detecting reagents. Rapid and efficient detection of active compounds can be achieved through bioautographic method. With this method, it is possible to localize antimicrobial activity on a chromatogram. Inhibition zones are observed directly on the TLC plate. The disadvantage of the method is that it requires more complex microbiological equipment.

2.7 Antioxidant and Cytoprotective Agents from Flowering Plants

2.7.1 Diseases Resulting from Oxidative Stress

Normal metabolism produces reactive oxygen species (ROS) which cause damage to DNA, proteins, and lipids hence ageing and neurodegenerative diseases (Ames et al., 1993). Reactive oxygen species (ROS) are generated by peroxisomes, mitochondria, and cell activation and are quickly neutralized by Glutathione (GSH) (Jatawa et al., 2012). However, exogenous sources like oxides of nitrogen in smoke, ultraviolet light, ionization radiation, environmental toxins and from metals and ions overcome glutathione (GSH) capacities in cells and oxidants are able to insult the cells which often undergo apoptosis (Noh & Ha 2011).

In fact, oxidative stress has been recognized to be involved in the diagnosis of several chronic conditions including cardiovascular diseases, cancer, diabetes,

arthritis, nephropathy and skin ageing (Ames et al., 1993; Jatawa et al., 2012). It is also the major cause for minor illness, fatigue, infertility and aging. Studies have recognized the involvement of ROS in the etiology of liver diseases such as hepatitis, fibrosis, cirrhosis, and hepatocellular carcinoma (Vitaglione et al., 2005). Liver is a vital organ which plays a major role in metabolism including glycogen storage, plasma protein synthesis, hormone production and detoxification of the body.

Antioxidants refer to any substance that delays, prevents or removes oxidative damage to a target molecule (Gutteridge & Halliwell 2010). Antioxidants comprise a broad and heterogeneous family of compounds that interfere with the oxidation of any oxidizable substrate (Halliwell & Gutteridge 2007). Antioxidants scavenge free radicals such as superoxides which accumulate in the body at a rate of 1 % per day at rest and increases 10-fold during exertion (Halliwell 1994). Consequently, mechanisms of protection of liver are compelling, and natural antioxidants are sought to counteract oxidative stress especially from flowering plants (Wiart 2006).

2.7.1.1Cancer

It is estimated that as much as 7.6 million deaths are caused by cancer worldwide in 2008. Cancer incidences in less developed countries are expected to increase 56 % in 2008 to more than 60 % in 2030 (Jemal et al., 2010). The

most common diagnosed cancer among men in Asia is lung cancer and breast cancer in women in most of the region worldwide (Jemal et al., 2010). Cancer is fundamentally a disease of regulation of cell and tissue growth and its development is a multi stage process which is termed carcinogenesis (Valko 2007).

Carcinogenesis can be described in three stages which are the initiation stage, promotion stage and the progression stage. ROS are claimed to act in all the three stages of carcinogenesis (Klaunig & Kamendulis 2004). A non-lethal mutation in DNA which produces altered cell is the initiation step of carcinogenesis. The mutation is then followed by one round of DNA synthesis to repair the damaged DNA produced. The promotion stage is where the clonal expansion of the initiated cells by the induction of cell proliferation or inhibition of apoptosis, thus results in the formation of identifiable focal lesion.

Progression is the final stage of carcinogenesis which involves cellular and molecular changes that occur from the preneoplastic to the neoplastic stage. ROS-induced DNA damage involves single or double stranded DNA breaks, purine, pyrimidine, or deoxyribose modifications, and DNA cross-links. DNA damage can result in either arrest or induction of transcription, induction of signal transduction pathways, replication errors, and genomic instability, all of

which is related to either one of the stages in carcinogenesis (Valko et al., 2006; Valko et al., 2007).

2.7.1.2 Neurodegenerative Diseases

An increasing number of proofs indicate a role of oxidative stress in several steps of the pathogenesis of many neurodegenerative diseases as changes related to oxidative stress have been observed in brain tissues and peripheral tissues from patients of Alzheimer's disease, Huntington's disease, Parkinson's disease and Amyotrophic Lateral Sclerosis (Migliore & Coppede 2009). Various neurodegenerative diseases have different symptoms, effect different part of the brains and have different causes. In fact, many of them are being studied on extensively and are being reviewed by excellent researchers. Halliwell (2006), have summarized the common affects and symptoms in the diseases which are impaired mitochondrial function, increased oxidative damage, defects in the ubiquitin-proteasome system, presence of abnormal and aggregated proteins, changes in iron metabolism and involvement of inflammation.

2.7.1.3 Ageing

The ageing process is a normal feature in the life cycle of all organisms, in which the functionality capacity of a variety of physiological systems deteriorates, attenuating the ability to maintain normal homestasis (Sohal et al.,

2002). A popular hypothesis of the cause of aging is accumulation of molecular oxidative damage induced by reactive oxygen species, which leads to senescence associated loss in physiological functions (Sohal 2002). Normal mitochondria metabolism produces ROS, as between 0.4 and 4 % of the oxygen consumed by the mitochondria is converted to ROS and will readily cause damage to biomolecules including DNA, protein and membrane lipids, thus define aging symptoms (Golden et al., 2002). The concept of ageing is supported by studies in many animals showing that ageing is associated with accumulation of oxidized forms of proteins, and it was proposed that ROS mediated protein damage is involved in the process (Valko et al., 2007).

The goal of ageing research is not to extend the lifespan of humans, but to improve and extend the functional lifespan of ageing individuals (Golden et al., 2002). As it is accepted as a normal cycle, therapeutic compounds are sought to maintain normal homeostasis of the body until the end of lifespan. Indeed, antioxidants are used either as functional food or incorporated to anti-aging formulations. Many natural product antioxidants are being sought and used as its safety and effectiveness is more trusted (Masaki 2010).

2.7.1.4 Inflammation

Inflammation is a spontaneous response of a normal body to any cellular or tissue injury to destroy and remove the cause of the injury, the injured tissues

and promote tissue repair. An uncontrolled response leads to excessive cellular and tissue damage that result in chronic inflammation and destruction of normal tissue. ROS are released by phagocytes to the sites of inflammation, thus causing the cell and tissue damage which includes apoptosis, and other chronic inflammatory diseases (Morcillo et al., 1999; Rahman & MacNee 2000).

2.7.2 Action Mechanism of Antioxidants

Gutteridge (1993) explained the different mechanisms of action of antioxidants which:

1. Removing oxygen or decreasing local oxygen concentrations
2. Removing catalytic metal ions
3. Removing key reactive oxygen species (ROS) such as O_2^- and H_2O_2
4. Scavenging initiating radicals such as OH^\bullet , RO^\bullet , RO_2^\bullet
5. Breaking the chain of an initiated sequence
6. Quenching or scavenging singlet oxygen.

Many antioxidants have more than one mechanisms of action. Aruoma (1994) classify antioxidants based on their mechanism of action, either as scavengers of free radicals, preventers of the formation of a radical or by repairing the damage caused. Relative importance of antioxidants *in vivo* relies on types of ROS that are generated, the source of generation and the type of environment it

is generated in. Hence, it is possible that an antioxidant may protect in one system but fail to protect, or may cause damage in another system (Halliwell et al., 1995).

2.7.2.1 Flavonoids

Flavonoids are known to display a wide range of pharmacological and biochemical properties and have long been recognized to possess anti-inflammatory, antimicrobial, anticarcinogenic, hepatoprotective and antioxidant activities (Kandaswami & Middleton 1997). Flavonoids are classed upon its structural differences to few classes which are flavones, isoflavonoids, chalcones, flavanones, flavanols and anthocyanins. Isoflavones were by far the most potent inhibitors of lipid peroxidation with the position of the singlet phenolic group (OH) in the chromane ring of alpha tocophenol corresponds to the 6-OH group of the isoflavonoids. A common feature of the active isoflavonoids is an ortho-dihydroxy benzene or catechol structure, which is considered to be important for antioxidative effectiveness of flavonoids.

Flavonoids can function as metal ion chelators and reducing agents, scavengers of ROS, chain-breaking antioxidants and quenchers of the formation of singlet oxygen. Protection of ascorbic acid appears to be one of their primary functions (Kandaswami & Middleton 1997). Flavonoids are antioxidant owing to their conjugated π -electron system which makes donation of electron or

hydrogen atoms possible from the hydroxyl moieties to free radicals (Gupta & Verma 2010). The antioxidant efficacy depends on their structural features such as the number and position of the hydroxyl moieties on the ring system and the unpaired electron in the oxidized phenolic intermediate (Gupta & Verma 2010).

Bors et al. (1990) suggested that there are three structural groups that are important determinants for radical scavenging and antioxidant potential which are:

- 1) The *O*-dihydroxy catechol structure in the B ring, which is the obvious target site for all flavonoids with a saturated 2, 3 bond
- 2) The 2,3 double bond in conjugation with a α -4-oxo function, which is responsible for electron delocalization from the B ring
- 3) The additional presence of both 3- and 5-hydroxyl groups for maximum radical scavenging potential and strongest radical absorption.

Flavonoids scavenge radicals effectively forming semiquinone free radical in alkaline solution. The semiquinone free radical or aroxyl radical may react with a second radical, acquiring a stable quinone structure (Figure 2.18). It is proposed that *O*-dihydroxyl structure in ring A makes better antioxidant than others (Tiwari 2001).

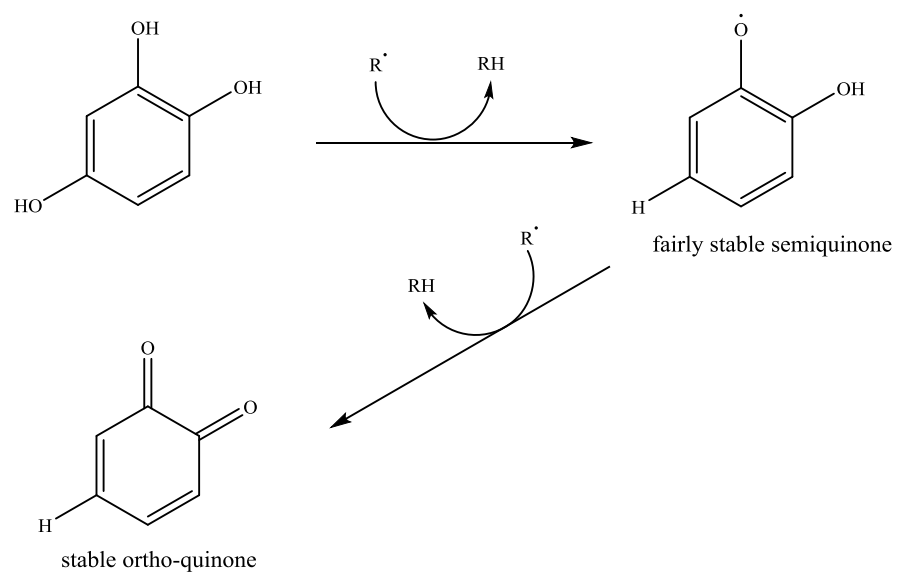


Figure 2.18. Scavenging of ROS (R^\bullet) by flavonoids and formation of a stable structure.

2.7.2.2 Alkaloids

Alkaloids contain one or more nitrogen atoms usually in combination as part of a cyclic system (Harborne 1984). A considerable number of alkaloids are specific to one family or few related plants. For instance, atropine from *Atropa belladonna* L. and colchicine from *Colchicum autumnale* L. (Harborne 1984). The presence of aromatic –OH group are partially responsible for their antioxidant efficiency, similar to phenolic antioxidants (Rackova et al., 2004).

A study on the structural aspects of alkaloids as antiradicals and antioxidants, which involves three alkaloids, berberine, jatrorrhizine and magnoflorine. (Figure 2.19) from *Mahonia aquifolium* (Pursh) Nutt. shows that alkaloids bearing unsubstituted –OH groups in jatrorrhizine and magnoflorine, proved to be able to scavenge free stable radical with the dihydroxylated alkaloid. Magnoflorine gives similar result to the standard used. Berberine, with absence of –OH groups showed only negligible activity which is in accord to the lack of any abstractable hydrogen (Rackova et al., 2004).

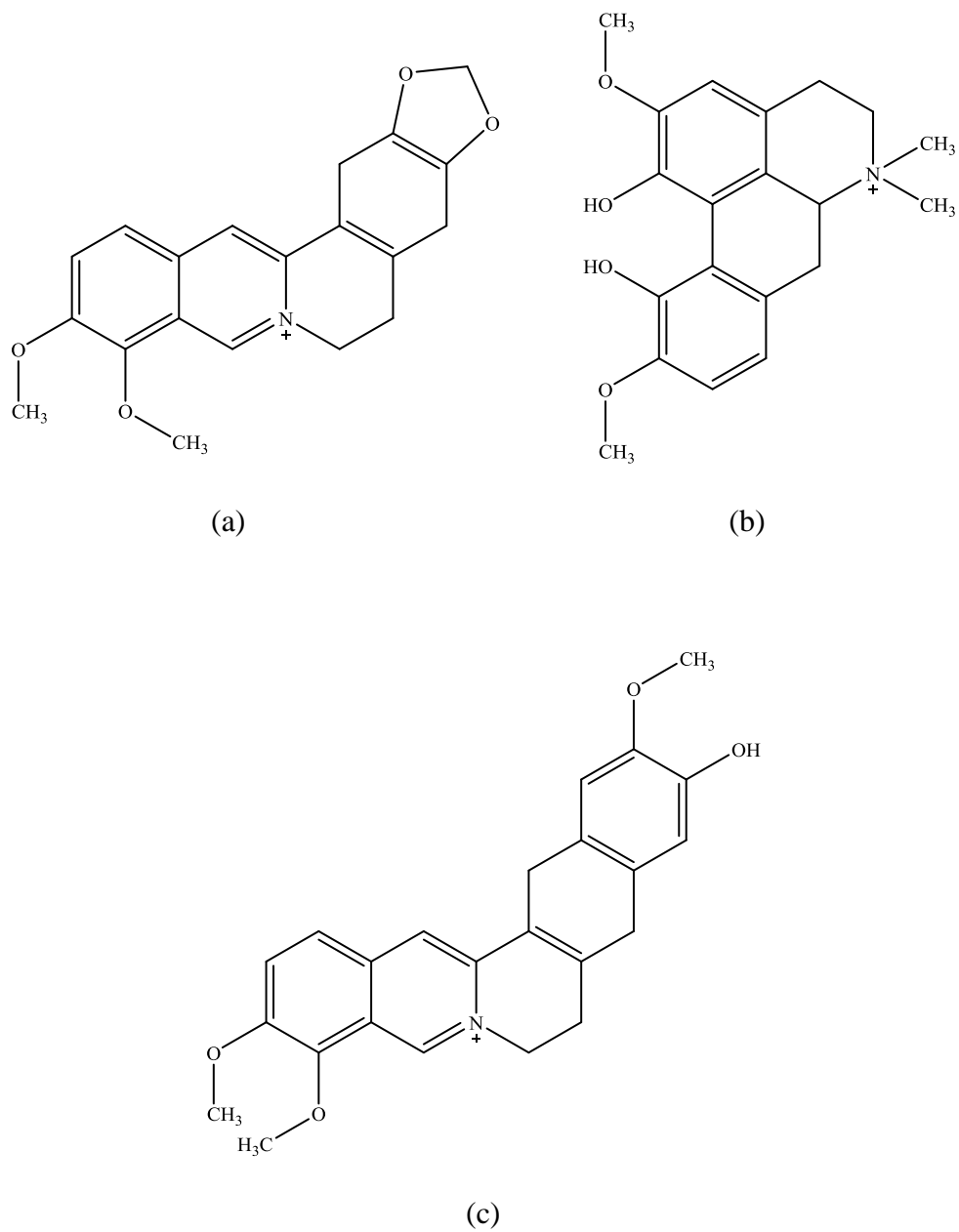


Figure 2.19. Alkaloids from *Mahonia aquifolium* (Pursh) Nutt. (a) Berberine, (b) Magnoflorine and (c) Jatrorrhizine.

2.7.2.3 Carotenoids

Carotenoids are C₄₀ tetraterpenoids which are widely distributed group of lipid soluble compounds. In plants, carotenoids have two essential functions which are as pigments in photosynthesis and as coloring matters in flowers and fruits (Harborne 1984). Although there are many identified carotenoids, but only a few are common in higher plants. Well known carotenoids are either simple unsaturated hydrocarbons lycopenes or their oxygenated derivatives xanthophylls. The chemical structure of lycopene (Figure 2.20a), comprise of eight long chain of isoprene units joined head to tail, which gives it a conjugated system of alternate double bonds, which is the chromophore. Common xanthophylls are either monohydroxycarotenes (lutein), dihydroxycarotenes (zeaxanthin) or dihydroxyepoxycarotenes (violaxanthin) (Figure 2.20b -c).

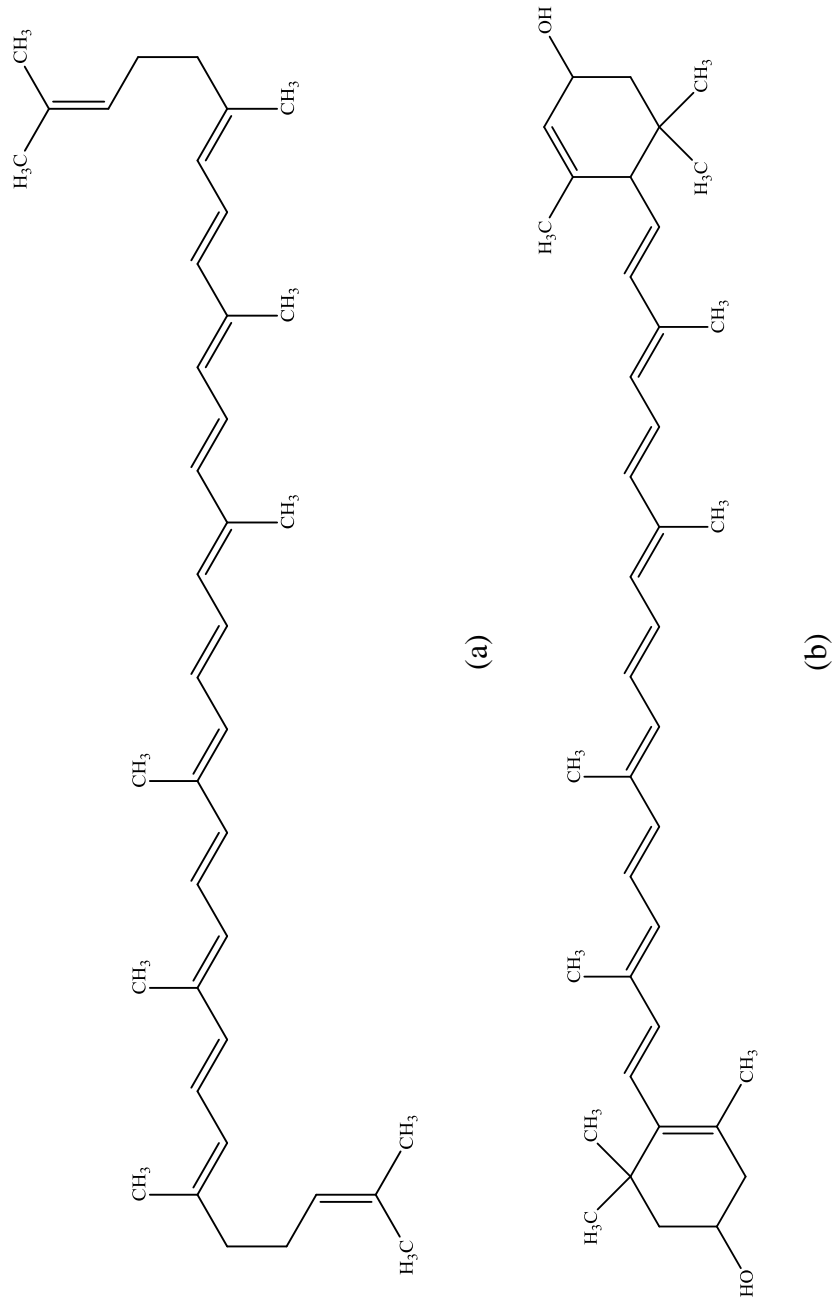
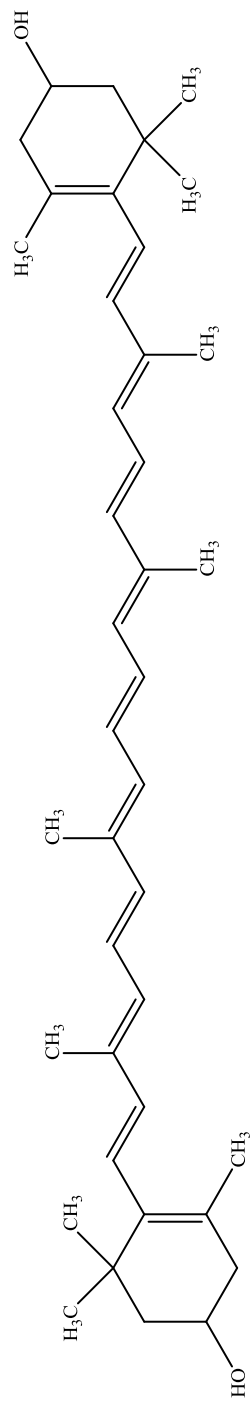
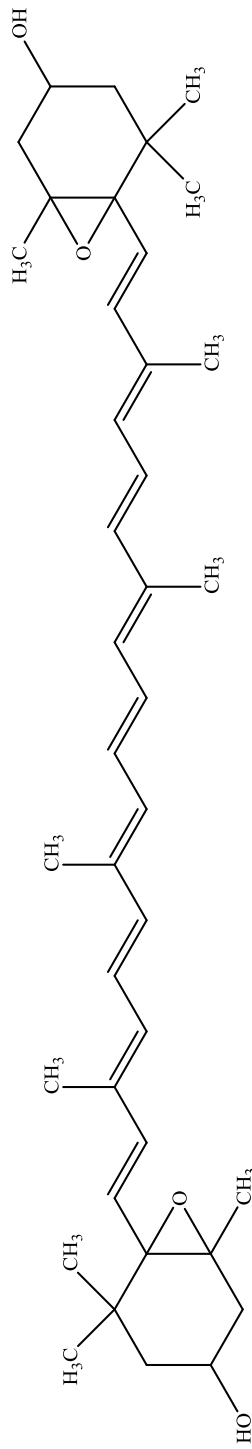


Figure 2.20 (a). Antioxidant carotenoids (a) Lycopene, (b) Lutein



(c)



(d)

Figure 2.20 (b). Antioxidant carotenoids (c) Zeaxanthin and (d) Violaxanthin.

Carotenoids are photoprotectants in both human and plant tissues, as they quench and inactivate ROS and become a radical themselves. Carotenoid radicals are stable owing to delocalization of unpaired electrons over the conjugated polyene chain of the molecules. This delocalization allows addition reaction to occur at many sites of the radical (Gupta & Verma 2010).

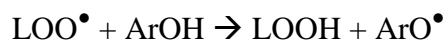
2.7.2.4 Phenolic acids and Tannins

Phenolic acids are antioxidant and studies showed that high antioxidant activity are present in molecules containing at least two neighboring phenolic hydroxyl groups (Cuppett et al., 1997). According to Dziedzic and Hudson (1984), a carbonyl group, such as an aromatic acid, an ester or a lactone, enhances the antioxidant activity of the phenolic acids. Besides that, steric hindrance of the phenolic hydroxyls by a neighboring methoxyl group also enhances antioxidant activity.

Phenolic acids inhibit lipid peroxidation by trapping the peroxy radical in one of the two mechanisms. In the first mechanism, the peroxy radical (LOO^\bullet) abstracts a hydrogen proton from the antioxidant (ArOH) to yield an aroxyl radical (ArO^\bullet) and the hydroperoxide (LOOH). In the second mechanism, a peroxy and an aroxyl radical react by radical-radical coupling to form a non

radical product (Cuppett et al., 1997). Both of the mechanisms can be pictured as below

Mechanism 1:



Mechanism 2 :



2.7.3 Tests to Assess Antioxidant Activity

As there are different mechanisms underlying each assays used and different applications for each, thus combination of different approaches and assays are needed to assess a potential antioxidant (Moon & Shibamoto 2009). Generally there are 2 general types of assays used; where one is an assay associated with lipid peroxidation i.e. β -carotene bleaching assay and another is associated with electron or radical scavenging property of the antioxidant i.e., ferric reducing antioxidant power (FRAP) assay and the diphenyl-picrylhydrazyl (DPPH) radical scavenging assay (Moon & Shibamoto 2009). According to Huang et al. (2005), to accurately estimate antioxidant potential of a sample, one or more method should be used covering the mechanism of different antioxidant reactions i.e., hydrogen atom transfer (ORAC) and electron transfer (FRAP and DPPH).

Free radical oxidation of lipid components in food is due to a chain of lipid peroxidations in fatty acids and their esters, which is responsible for the off-flavour in foods (Aruoma 1994). Lipid peroxidation is initiated by ROS as it subtracts a hydrogen atom from a methylene group of an unsaturated fatty acid and subsequently form free radicals such as peroxy radical. Once the radicals are formed, lipid peroxidation progresses and produce various secondary oxidation products. Most antioxidant tests associated with lipid peroxidation involves monitoring of hydroperoxides or a specific oxidative secondary product (Moon & Shibamoto 2009).

2.7.3.1 β -carotene Bleaching Assay

In the β -carotene bleaching assay used in this study, the reducing amount of β -carotene (radical scavenger) is measured spectrophotometrically at 470 nm as it scavenges peroxy radical (LOO^\bullet) formed by linoleic acid (lipid) in presence of ROS and O_2 to form a stable β -carotene radical (Figure 2.21). If an antioxidant is present in a testing solution, it reacts competitively with the peroxy radical. Therefore, antioxidant effects are monitored with the bleaching of the test solution which is the reducing amount of β -carotene.

2.7.3.2 2,2-diphenyl-1-picrylhydrazyl (DPPH) Radical Scavenging Assay

The DPPH radical scavenging assay implies the theory that an antioxidant is a hydrogen donor, by which DPPH^\bullet accepts hydrogen from an antioxidant. The

antioxidant effects are proportional to the reducing amount of DPPH[•] in test samples. An easy monitoring of the DPPH[•] is able to be performed as it gives strong absorption maximum at 517 nm. The color turns from purple to yellow following the formation of a stable DPPH upon absorption of hydrogen from an antioxidant (Figure 2.23). This assay is quite popular in natural products studies as it is simple and highly sensitive.

2.7.3.3 Ferric Reducing Antioxidant Potential (FRAP) Assay

The FRAP assay is associated with electron transfer. When a Fe³⁺-TPTZ complex is reduced to Fe²⁺ form by an antioxidant under acidic conditions, an intense blue color is observed which is monitored at 593 nm (Figure 2.22). Therefore the antioxidant effect is measured by the reducing capability of an antioxidant. The FRAP assay gives fast reproducible results but has to be used in an aqueous system and the antioxidants must be water-soluble.

2.7.4 Cytoprotection and Cytotoxicity

Cytotoxic agents are agents which damages cell at a certain concentration. Cytoprotective agents are natural or artificial substances which promote defense mechanism and do not have curative effects. They can stimulate production of natural cell defenses against cell insults (Meerof 1985). Cytoprotection are important to protect cells from ROS and oxidative damages that will damage DNA, lipids and proteins intracellular, thus will lead to illness

and conditions as mentioned above. Cytoprotection mechanism of antioxidants includes (Gutteridge 1993):

1. Preventing radical formation
2. Intercepting formed radicals
3. Repairing oxidative damage
4. Increasing elimination of damaged molecules
5. Non repair recognition of excessively damaged molecules in order to prevent mutations occurring.

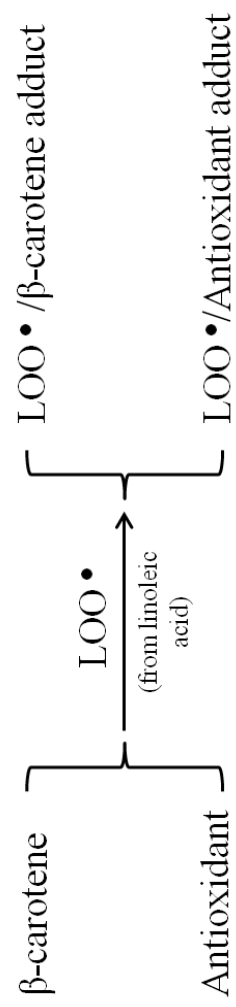


Figure 2.21. Mechanism of β -carotene bleaching antioxidant assay.

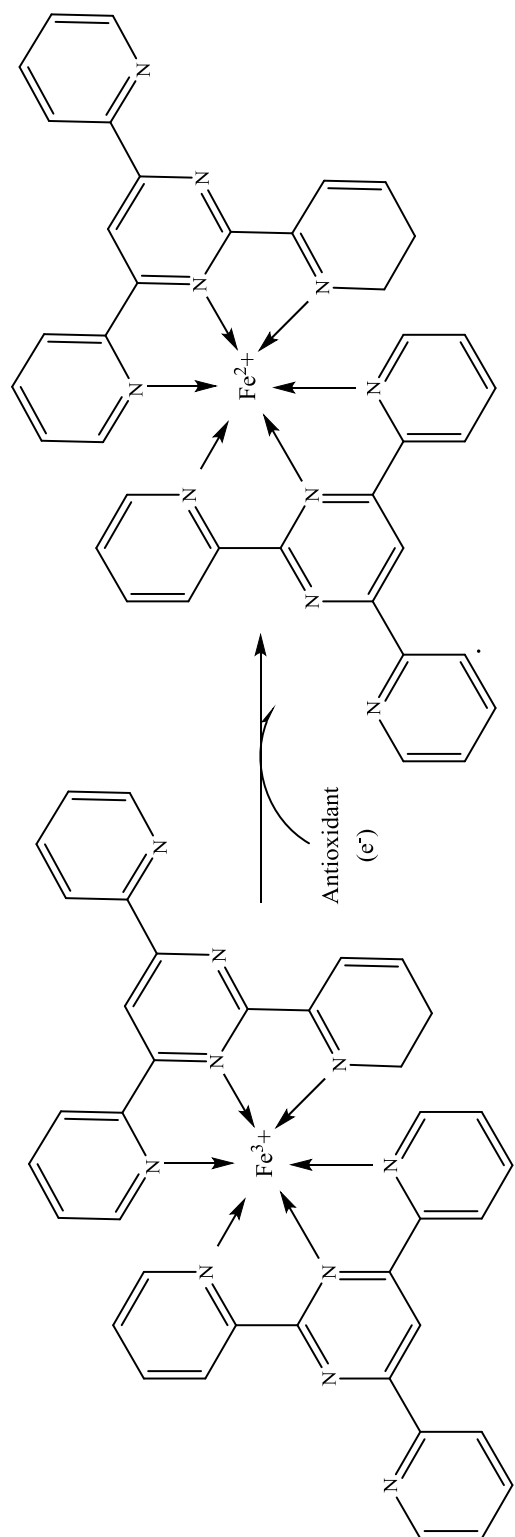


Figure 2.22. Mechanism of Ferric Reducing Antioxidant Power (FRAP) assay. Change from Ferric tripyridyltriazine (Fe^{3+} -TPTZ) complex (Left) to Ferrous tripyridyltriazine (Fe^{2+} -TPTZ) complex (Right) which gives an intense blue colour.

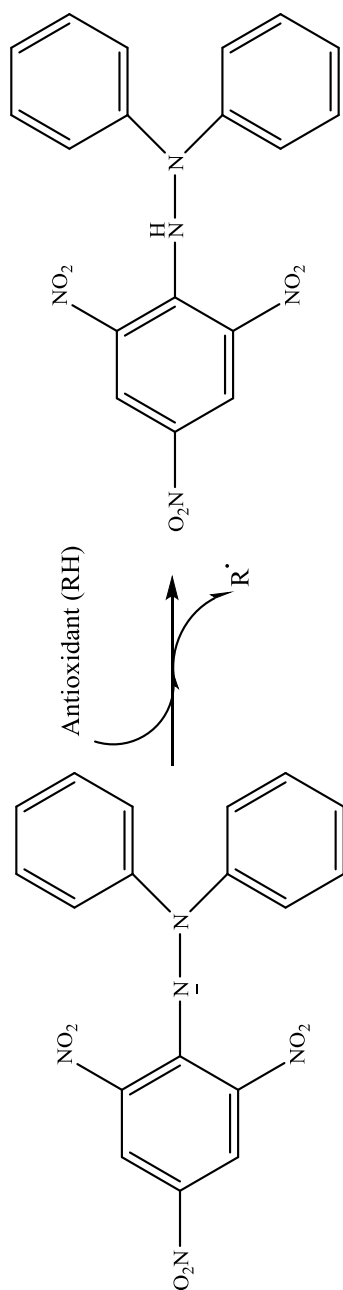


Figure 2.23. Mechanism of the DPPH radical scavenging assay. Change from 2,2 Diphenyl-1-picrylhydrazyl free radical (DPPH[•]) to 2,2-Diphenyl-1-picrylhydrazyl (DPPH)

2.7.5 Tests to Assess Cytoprotection and Cytotoxicity

Cytotoxicity is assessed by methods that observe cell viability and proliferation in cell culture. There are a number of methods that are developed in which most have been optimized using the 96 well plates and are colorimetric and luminescence based (Wayermann et al., 2004). Different parameters associated with cell death and proliferation are the basis of the methods developed which includes measurement of the cytoplasmic enzyme released i.e., lactate dehydrogenase (LDH) (Korzeniewski & Callewaert 1983), metabolic activity of viable cell i.e, reducing of tetrazolium salts in MTT (Smith 1951) and neutral red assay (DeRenzis & Schechtman 1973), and presence of adenosine triphosphate (ATP) (Crouch et al., 1993).

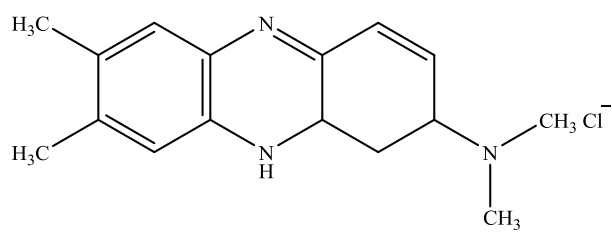
In this study, the neutral red uptake assay was used to analyze cytotoxicity and cytoprotective properties of the samples. The neutral red uptake assay is one of the most commonly applied cytotoxicity tests with many applications. The uptake of neutral red depends on the cell's capacity to maintain pH gradients, through the production of ATP. At physiological pH, the dye is present at a net charge close to zero which enables it to penetrate through the membranes of the cell. When the cell dies, or the pH gradient is reduced, the dye cannot be

retained. Consequently, the amount of retained dye is proportional to the number of viable cells (Repetto et al., 2008).

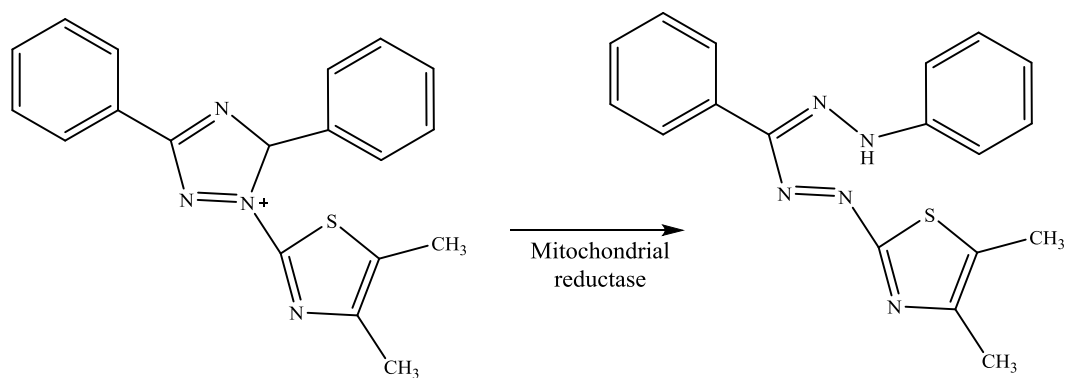
MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) is a water soluble tetrazolium salt. The MTT assay is based on the reduction of the soluble yellow MTT tetrazolium salt to a blue insoluble MTT formazan product by mitochondrial succinic dehydrogenase (Mosmann 1983). MTT assay is used with tumor cells and fibroblast cell lines to evaluate cytotoxicity of chemotherapeutic agents (Borenfreund et al., 1988). In a study comparing 3 cytotoxicity assays (Fotakis & Timbrell 2006), the MTT assay and the neutral red uptake assay being the most sensitive assay, showing statistically difference between treated cells and the controls. Both assays appear to be more sensitive in detecting early toxicity compared to LDH leakage assay.

2.7.6 Current Antioxidant and Cytoprotective Agents from Flowering Plants

As the demand for antioxidant and cytoprotective agents rises for the treatment or preventive measures from oxidative stress conditions, natural sources are sought as an alternative as the natural and herbal sources are non-toxic with proven therapeutic benefits and has been used since ancient times (Paul et al., 2011). Today, more than 70 % of the world population still depends on plant sources to meet their health care needs. One of the most important plant sources



(a)



(b)

Figure 2.24. (a) Neutral red dye, (b) MTT salt (Left) changes to MTT formazan product (Right).

with active properties are flowering plants (Wiart 2006). Flowering plants are rich sources of polyphenols which includes anthocyanins, stilbenes, flavonoids, tannins and lignins, which are known for its antioxidant and cytoprotective properties (Wiart 2012).

Among known antioxidant and cytoprotective agents from flowering plants are quercetin, thymol and carvacrol from *Origanum onites* L. (Ozkan & Erdogan 2010). Resveratrol is present in seeds of grapes, in peanuts and berries and has profound antioxidant, anti-inflammatory and antiproliferative property. It is used in topical applications for protection from Ultraviolet B (UVB) induced lipid peroxidations (Svobodova et al., 2003) (Figure 2.25). Epigallocatechin gallate (Figure 2.26) from green tea (*Camellia sinensis* L.) is responsible for its antioxidant and anticancer effects (Chen et al., 1998). Curcumin and bis-demethoxycurcumin from *Curcuma longa* L. are examples of antioxidant and cytoprotective compounds which has been observed to exhibit *in vitro* and *in vivo* effects (Figure 2.27). Lately, the pharmacokinetics and pharmacology aspect of the compounds has been studied and are going through clinical human trials (Hatcher et al., 2008).

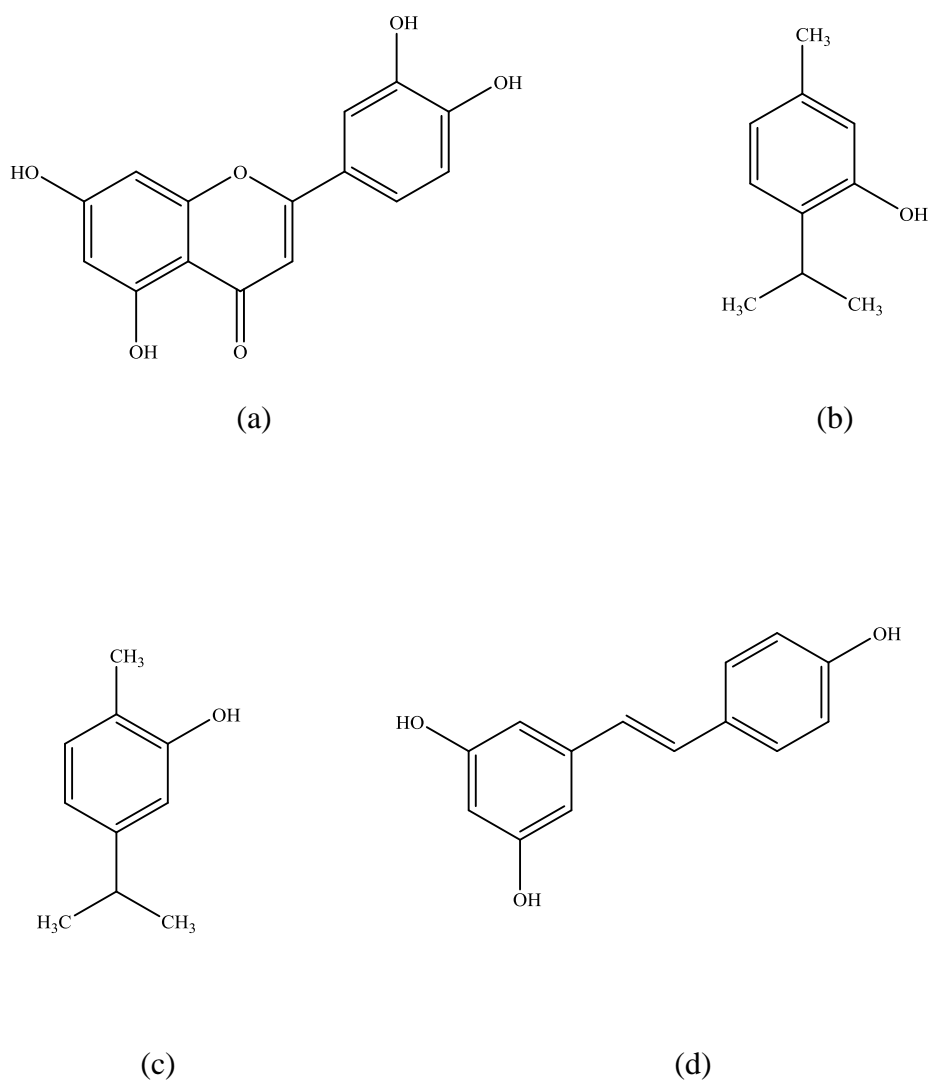


Figure 2.25. Antioxidant and cytoprotective agents from flowering plants (a) Quercetin, (b) Thymol, (c) Carvacrol, (d) Resveratrol,

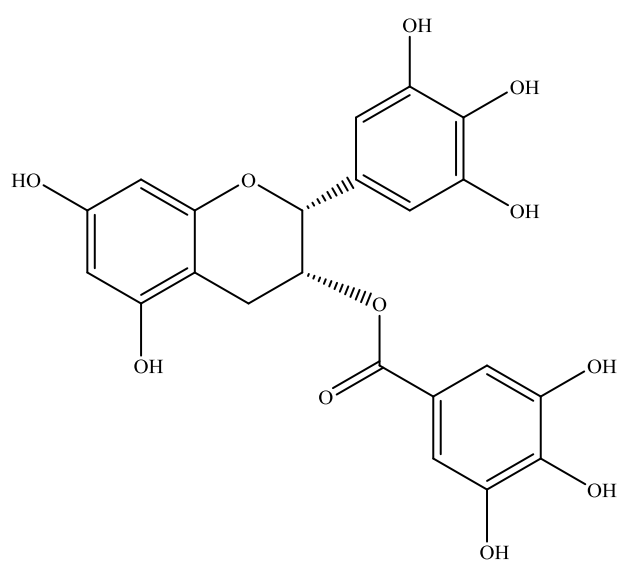
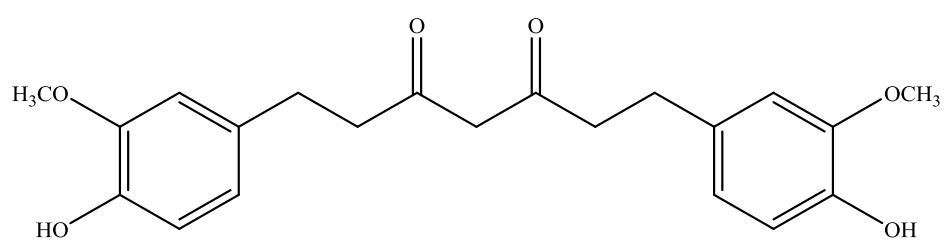
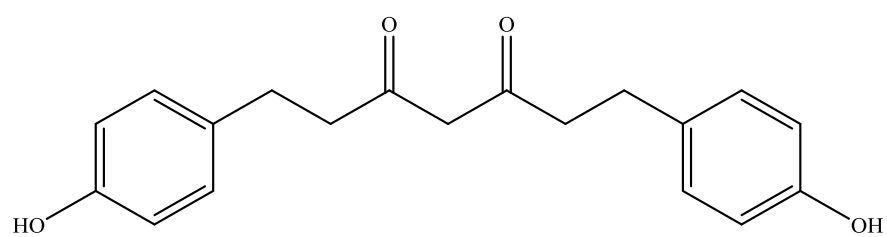


Figure 2.26. Epigallocatechin gallate.



(a)



(b)

Figure 2.27. Bioactive compounds from *Curcuma longa* L. (a) Curcumin, (b) Bis-demethoxycurcumin.

2.8 Approaches in Natural Product Research

Indeed, the demand for antibacterial and antioxidant agents are high as cure for these illness and diseases that are caused by bacterial infections and oxidative stress are still in need, and the most potential source would still remain to be sought from plants. This leads to extensive development in the natural product research field. Different approaches are being implemented by different research groups in aiming to identify cures from the nature and preparing it to be finally used at the patients' bedside.

One of the approaches that are being implemented is random selection of plants followed by classical chemical screening. Applying this method, the plants are randomly selected and screened for their biological activities with simple bioassays. The active plant extract will then be screened for the absence and presence of a certain group of compounds with classic phytochemical methods. Although the tests are simple to perform, it is usually difficult to relate the presence of the compounds with the biological activity that it illicit.

One other common approach that is becoming more popular is the bioassay guided fractionation. Scientist applying this approach would choose the plants with ethnotraditional applications. The extract would then be screened for its activity with a certain bioassay. The active extract would then be fractionated

with any means of chromatography and the resulting fractions would then be screened again using the same bioassay. The cycle of method will be continued until the active constituents are located. Although this approach is more directed and focused, it consumes a lot more time and more laborious. Indeed, this approach has resulted in isolation of many compounds to date.

In this study, we have opted to use the bioassay guided fractionation approach. The plant has been chosen from literature review of its traditional uses and previous studies done either in our own lab or other labs. We fractionated the active extract and assessed the activity of the fractions with bioassays. The active fraction is then further assessed with more specific bioassays and its major constituent isolated and elucidated. Other constituents within the fraction were identified by comparing the fragments generated by LC-MS with the literature present.

CHAPTER 3

MATERIALS AND METHODS

3.1 Instruments

- Grinder

The dried plant materials were grounded with a heavy duty grinder (Waring, USA) into powder form.

- Rotary evaporator

Extracts and other forms of liquid samples were concentrated using rotary evaporator (Buchi, USA) at the range of room temperature to not more than 40 °C.

- Freeze drier

Water extracts and fractions isolated with a high water content was dried using a freeze drier (Christ Alpha, Germany).

- Spectrophotometer UV-Vis

Total phenolic contents were conducted using a UV-Vis spectrophotometer from Biochrom Libra S12 (USA).

- Microplate reader

All absorbance based antioxidant, antibacterial and cytoprotective assays using 96 well plates were measured using Varioskan Flash microplate reader from Thermo Fisher (USA) and result were tabulated with ScanIt Software.

- High-performance liquid chromatography (HPLC)

HPLC analysis of the samples were done with Varian 940-LC (USA) equipped with a binary pump, a photodiode array (PDA) detector and fraction collector. This HPLC set-up was also used to scale-up separations from analytical scale to semi-preparative level in order to perform collections of the samples separated. Column used for analytical separations was Zorbax SB-C18 from Agilent Technologies (USA) with internal diameter of 4.6 mm, length 150 mm and particle size of 5 micron. For semi-preparative scale up, the same column was used with internal diameter of 9.4 mm.

- Mass spectrophotometry (MS)

MS analysis was performed on a LC-MS system (Bruker MicroTOF Q, Germany). Analysis was performed in Universiti Kebangsaan Malaysia (UKM). All scan analyses were performed from m/z 150 – 1500. Column used was the same column as analytical separations with HPLC. Conditions were

ESI negative mode, capillary 3000v, collision cell RF 250.0 Vpp, nebulizer 0.5 bar, dry heater 180 °C, dry gas 5.0 L/min.

- **Field Emission Scanning Electron Microscopy (FESEM)**

Surface morphology of the bacterial cells were observed and imaged with FEI Quanta 400 FESEM. All images are captured with ESEM vacuum wet mode at 20 000 kV of voltage. Magnification used was 30 000x unless stated otherwise in result.

- **Nuclear Magnetic Resonance (NMR)**

NMR analyses of the compound(s) were using 600 Ultrashield from Bruker (USA) at University of Nottingham UK Campus.

3.2 Materials

Solvents: Hexane, chloroform, ethyl acetate, ethanol, methanol and dimethylsulfoxide (DMSO) are all of analytical grade unless specified. Acetonitrile for HPLC is of HPLC grade. Water used is of distilled water and Ultrapure water (PURELAB Flex, Elga, UK) for HPLC.

Chemicals: DPPH, TPTZ, Trolox, Quercetin. Ascorbic acid, linoleic acid, Tween 80, FeCl₃, FeSO₄, Folin-Ciocalteu reagent, β-carotene, Sephadex LH-

20, propidium iodide, Nisin, ATP, Nigericin, glucose, valinomycin, DiSC₃(5), MTT and t-BHP were purchased from Sigma, Germany. Gallic acid was purchased from TCI, Japan. CellTiter Glo kit was purchased from Promega, USA.

Antibiotics: Streptomycin, tetracycline and amphotericin B were from Sigma, Germany. Ampicillin was purchased from Amresco, USA.

Media: Mueller-hinton broth (MHB), Mueller-hinton agar (MHA) and potato dextrose agar (PDA) were purchased from Hi-Media Labs, India. Minimum Essential Eagle Media (MEEM), Fetal bovine serum and phosphate buffer saline were purchased from GIBCO, USA.

Bacteria, fungal and cell strains: *Bacillus cereus* (ATCC 11778), *Bacillus subtilis* (ATCC6633), *Staphylococcus aureus* (ATCC 11632), *Escherichia coli* (ATCC 8739), *Pseudomonas aeruginosa* (ATCC 10145), *Citrobacter freundii* (ATCC 809) were all purchased from American Type Culture Collection, USA. Methicillin-resistant *Staphylococcus aureus* (MRSA) were obtained from the Department of Medical Microbiology, UKMMC.

Formulation excipients: Emulsifying wax, liquid paraffin, white soft paraffin, arachis oil, oleic acid, wool fat, glycerol, cetostearyl alcohol and sodium

stearate were all purchased from R&M Chemicals, India. Calcium hydroxide was purchased from Sigma, Germany.

3.3 Plant Collection and Extraction

3.3.1 Extraction

Acalypha wilkesiana var. *macafeana* hort. was collected at Jalan Broga, Semenyih (2°56'43" North, 101°56'44" East) and authenticated by the Forest Research Institute Malaysia (FRIM). The plant was collected in March 2009. Aerial part of *A. wilkesiana* i.e., leaves, stems and flowers, without roots, were dried at room temperature (27 °C). After it was dried, the plant was grinded into powder. The secondary metabolites from the plant were then extracted with successive sequential maceration in solvent starting from a non-polar solvent to a polar solvent which was hexane, ethyl acetate, ethanol and water in sequence. Extraction was done with each solvent for 3 subsequent days with 1:8 ratio of sample to solvent. All the plant extracts were then concentrated using the rotary evaporator at 40 °C, and kept in the dessicator until dried. The water extract was further dried using a freeze drier to remove all water molecules that might be present in the extract. Once dried, all the extracts were kept at -80 °C prior to use.

3.3.2 Fractionation and isolation

The ethanol extract of *A. wilkesiana* var. *macafeana* hort. was then further fractionated by column chromatography. Fractionation was done according to Adesina (2000) with slight modification. The column (2.5 cm i.d and 40 cm length) was prepared using Sephadex LH-20 (25 g) preswollen in 25 % aqueous ethanol overnight. The ethanol extract was weighed and dissolved in 25 % aqueous ethanol. The dissolved extract was filtered (0.45 µm, nylon) before loaded into the packed column. Elutions were done with gradient increase of ethanol in water starting from 25 % aqueous ethanol, 50 % aqueous ethanol, 75 % aqueous ethanol, 95 % ethanol, and finally with 70 % aqueous acetone. The elution afforded a total of 5 fractions i.e., F1-F5. Fractions were dried with the rotary evaporator with temperature of the water bath not exceeding 40 °C. The fractions were then used for further assays and analysis throughout the research.

3.4 Chemical and Biological Assay on Fractions

3.4.1 Antioxidant screening

3.4.1.1 TLC Screening with DPPH Spray

The fractions were qualitatively assessed for their antioxidant properties by spraying with DPPH spray prepared with methanol (0.2 % DPPH in methanol). This method is a preliminary screening for metabolites with antioxidant

property, and can be done very fast. The TLC plate that has been developed were left to dry, then sprayed with the DPPH spray and left for thirty minutes. Active antioxidant compounds appeared as yellow spots against a purple background (Braca et al., 2002).

3.4.1.2 Ferric reducing antioxidant power (FRAP) assay

The antioxidant capacity was determined following the procedure described by Benzie and Strain (1996) with several modifications. FRAP reagent were prepared fresh by adding 10 mM TPTZ (dissolved with 40 mM HCl), 20 mM FeCl_3 in water and 300 mM of acetate buffer in ratio of 1:1:10. Samples and standards were prepared at a concentration of 1 mg/mL. Briefly, 20 μL of sample were transferred into wells and subsequent dilutions were done on each sample until five different concentrations were obtained. 180 μL of FRAP reagent were added in each well making the final volume to 200 μL . Sample colour corrections were done to subtract off absorbance from the sample due to its colour by making a blank devoid of sample and solvents. The microtiter plates were then incubated at 37 °C for 90 minutes before absorbance were recorded at 600 nm. Trolox and quercetin were used as positive control. The absorbance was compared to a FeSO_4 standard curve and results were expressed as mole Fe (II)/g sample.

3.4.1.3 DPPH radical scavenging assay

The antioxidant activity was also studied through the free radical scavenging activity of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical. The assay was conducted on the fractions with reference to Alothman (2009) with slight modifications. Samples and ascorbic acid were prepared in the concentration of 1 mg/mL. 10 µL of samples and ascorbic acid were transferred from stock solution of 1 mg/mL into micro wells. Ten fold dilutions of each sample and ascorbic acid were done to attain 5 different concentrations. 200 µL of methanolic DPPH (50 µM) was added into the tubes, and then thoroughly mixed before incubated in the dark at room temperature for 30 minutes. The absorbance was measured at 517 nm, against a blank methanol without DPPH. Colour corrections were done by measuring the absorbance of the samples and solvents and its absorbance subtracted from the absorbance measured at the end of the assay. This was done to make sure that the absorbance of the samples does not interfere with the results. Results were expressed as IC₅₀ concentration where 50% inhibition of the DPPH radical was obtained. Percentage of inhibition of the DPPH was calculated following the equation:

$$\% \text{ inhibition of DPPH} = \left[\frac{\text{Abs control} - \text{Abs sample}}{\text{Abs control}} \right] \times 100$$

where Abs control is the absorbance of DPPH solution without sample. Ascorbic acid was used as an antioxidant standard.

3.4.1.4 β -carotene Bleaching assay

To evaluate the antioxidant activity related to lipid peroxidations, the β -carotene bleaching assay was applied with reference to Barreira (2008). A solution of β -carotene was prepared by dissolving 2 mg of β -carotene in 10 mL of chloroform. 2 mL of this solution was pipetted into a 100 mL round bottom flask. Added into the round bottom flask were 40 mg of linoleic acid and 400 mg of Tween 80 emulsifier. The chloroform was then removed using the rotary evaporator at 40 °C. After all the chloroform was removed, 100 mL of distilled water was added and the flask was then shake vigorously. 240 μ L of the β -carotene solution was transferred into each well containing 10 μ L of sample.

The t_0 absorbance (absorbance at 0 min) was taken immediately after the plates were ready. Incubations were done at 50 °C for 4 hours or until the control sample had changed colour. A control well was prepared containing water and the β -carotene solution only. Blank devoid of β -carotene were also prepared for each sample to correct for the colour of the sample. Oxidations of β -carotene emulsion were monitored using a spectrophotometer by measuring the absorbance at 490 nm. Degradation rates were calculated according to the first-order kinetics with reference to Mayachiew (2008) using the below formula:

$$\text{Sample degradation rate (DR)} = \ln a/b \times 1/t,$$

where a is the initial absorbance at t_0 ; b is the absorbance at 240 min; t is time (min).

The antioxidant activities were expressed as % inhibition relative to the control using:

$$\text{Antioxidant activity} = \frac{\text{DR Control} - \text{DR Sample}}{\text{DR Control}} \times 100$$

Results were expressed as EC₅₀ concentration where 50 % degradation of the β-carotene was obtained.

3.4.1.5 Determination of total phenolic content

Total phenolic contents of the fractions were determined using Folin-Ciocalteu assay adapted from Singleton (1999) and Waterhouse (2001). Two μL of sample was mixed with water (158 μL) and Folin-Ciocalteu reagent (10 μL) and left to stand for 8 minutes in room temperature before 30 μL of sodium carbonate solution was added in. This makes the total volume 200 μL. The solution was mixed and allowed to stand for 30 minutes in the water bath at 40° C. Then, the absorbance was recorded at 700 nm using the microplate reader. A calibration curve of gallic acid was also prepared starting with final concentration of 50 μg/mL and subsequent 2 fold dilution. Results were expressed as g of gallic acid equivalent (GAE)/100 g sample.

3.4.2 Cytotoxic and cytoprotective evaluation

3.4.2.1 Cell culture

All the cytotoxic and cytoprotection assays were executed with cell culture with the conditions as stated. HepG2 cells were grown to 90% confluence before seeding into 24 - well culture plates, and left overnight to adhere prior to treatment at 37°C in a 5 % CO₂. Cells were grown in Minimum Essential Eagle medium with 10 % v/v foetal bovine serum (FBS), 1 % v/v non-essential amino acid solution, 2 µg/mL of fungizone and 0.05 mg/mL of gentamicin.

3.4.2.2 Neutral red cytoprotection assay – direct activity

Cells were treated with the ethanol extract, the F5 fraction or quercetin at various concentrations for 5 hours, in the absence or presence of 0.5 mM t-BHP, in medium containing 2 % v/v FBS. The medium was washed off, and replaced with complete medium containing neutral red at 20 µg/mL. Cells were incubated at 37°C for one hour. Medium was removed again and cells were washed with buffered saline prior to fixation of the cells with water:ethanol:glacial acetic acid (50:50:1 v/v). Plate was shaken on an orbital shaker for 10 mins to solubilize the cell-retained dye. Absorbances were taken at 540 nm, from which cell viability was calculated.

3.4.2.3 Neutral red cytoprotection assay – indirect activity

Cells were treated with the ethanol extract, the F5 fraction or quercetin at various concentrations for 20 hours in complete culture medium. The medium was removed and cells were exposed to 0.5 mM t-BHP in medium containing 2 % v/v FBS for 5 hours. The medium was washed off, and replaced with complete medium containing neutral red at 20 µg/mL. Cells were incubated at 37°C for one hour. Medium was removed again and cells are washed with buffered saline prior to fixation of the cells with water:ethanol:glacial acetic acid (50:50:1 v/v). Plate was shaken on orbital shaker for 10 mins to solubilize the cell retained dye. Absorbances were taken at 540 nm, from which cell viability was calculated.

3.4.3 Antimicrobial screening

3.4.3.1 Pour plate disc diffusion assay

Samples were screened for their antibacterial activity using the disc diffusion assay of Kelmanson et al. (2000). Samples were dissolved in dimethyl sulfoxide (DMSO). The test microorganisms used were Gram-positive *Bacillus cereus* ATCC 11778, *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 11632 and Methicillin resistant *Staphylococcus aureus* (MRSA) clinical strain and Gram-negative *Escherichia coli* ATCC 8739, *Pseudomonas aeruginosa* ATCC 10145, and *Citrobacter freundii* ATCC 8090. Bacteria were grown in Mueller-Hinton broth until specific optical density (OD) that will

give 1×10^8 bacteria/mL. Mueller-Hinton soft agar was prepared by using half of the amount of agar needed to prepare the Mueller-Hinton agar.

Six mL of molten Mueller-Hinton soft agar inoculated with 100 μ L of bacteria were poured on a prepared Mueller-Hinton agar plate and quickly swirled to get a confluent distribution of bacteria. Discs were prepared with 100 μ g dose of samples and 5 μ g of positive standard. Ampicillin and streptomycin were used as positive controls and DMSO as negative controls. Plates were incubated at 37 °C overnight. The antimicrobial activity was expressed as the mean diameter of the inhibition zone (mm) including diameter of disc (6 mm). Diameters were measured manually with a caliper.

3.4.3.2 Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

Minimal inhibitory concentration (MIC) and bactericidal (MBC) concentration were determined for the samples showing antibacterial activity in the disc diffusion assay. These were determined by the microbroth dilution method as of Kelmanson et al. (2000) with modifications. The broth dilution method was employed on Gram-positive *Bacillus cereus* ATCC 11778, *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 11632 and Methicillin resistant *Staphylococcus aureus* (MRSA) clinical strain. Two fold serial dilutions of the samples were used ranging from 3 mg/mL to 0.0234 mg/mL. Antibiotics used

were streptomycin (50-0.391 $\mu\text{g/mL}$) and ampicillin (100-0.781 $\mu\text{g/mL}$). MIC values were taken as the lowest concentration of samples that inhibited bacterial growth after 18 hours incubation at 37°C. MBC were determined by dispensing 10 μL of the broth of the MIC concentration and plating on MHA plate. Plates with growth less than 10 colonies after 18 hours incubation were considered the bactericidal concentration.

3.5 Chemical and Biological Assays on the Active Fraction

3.5.1 HPLC profiling of F5

Compounds in F5 were separated by HPLC with a Zorbax C₁₈ column (4.6 mm x 150 mm, 5 μm particle size) from Agilent Technologies with the mobile phase gradient as in Table 3.1. Peaks were detected at dual wavelength, 254 nm and 280 nm. The UV spectra of all the peaks were scanned using a PDA detector, to identify the λ maximas of the compounds concurrently. Injection volume for analytical separation was 20 μL .

Table 3.1 Mobile phase gradient for analytical separations of F5.

Time	Flow rate	Mobile phase A (Water)	Mobile phase B (Acetonitrile)
Prerun	1.0	90	10
3.0	0.5	90	10
35.0	0.5	60	40
40.0	1.0	5	95
43.0	1.0	5	95
43.1	1.0	90	10
48.0	1.0	90	10

3.5.2 Identification of the constituents in F5 by TOF-MS

The identities of the compounds in F5 were screened by LC-MS profiling with Bruker MicroTOF II system. Sample was dissolved with 20 % acetonitrile and separation of the constituents was performed on a Zorbax C₁₈ column (4.6 mm x 150 mm, 5 µm particle size) from Agilent Technologies with gradient acetonitrile and water mixtures. The peaks were detected at 280 nm. All the peaks were then diverted to the MS system and scanned from m/z 100 – 1500 for detection of the fragments. All MS analysis were performed in negative ionization mode.

3.5.3 Isolation of the major constituent of F5

Isolation of the major compound in F5 was done by up-scaling the HPLC system to a semi-preparative system by increasing the flow rate, detector flow cell and using a semi-preparative column (Zorbax SB-C₁₈, 9.4 mm x 150 mm, 5 µm particle size) from Agilent Technologies. The scale-up method is visualized in Table 3.2. The fraction collector was set to collect upon threshold at 0.5 mins for every tube. All the fractions collected were dried using a freeze drier for further analysis. Injection volume for semi-preparative separation was 100 µL.

Table 3.2. Mobile phase gradient for scale-up semi preparative separations.

Time	Flow rate	Mobile phase A (Water)	Mobile phase B (Acetonitrile)
Prerun	4.2	90	10
3.0	2.1	90	10
35.0	2.1	60	40
40.0	4.2	5	95
43.0	4.2	5	95
43.1	4.2	90	10
48.0	4.2	90	10

3.6. Formulation of F5 as a Topical Preparation

3.6.1 Formulation of the topical preparation

Three bases of formulations were prepared. The formulations used were: formulation 1 of soft paraffin base, formulation 2 of wool fat cream base and formulation 3 of liquid paraffin base. The excipients contained in each formulation with the percentage are detailed in Table 3.3. The tannin fractions were dissolved in the water percentages of the formulation at an MBC concentration. 18 mg of the tannin fraction (F5) were incorporated in 5 g of the cream bases prepared. The following commercial antibacterial products were used as positive control: Burnol[®] and Betadine[®]. The active ingredients in Burnol[®] are aminacrine HCl, cetrimide and thymol, where as in Betadine[®], the active ingredient is providone iodine. The cream bases were used as negative controls (placebo).

Table 3.3. Excipients contained in the formulations

Formulation	Excipients	Percentage (%)
Formulation 1	Emulsifying wax	10
	Liquid paraffin	10
	White soft paraffin	20
	Water	60
Formulation 2	Arachis oil	32
	Oleic acid	0.5
	Calcium hydroxide	0.045
	Wool fat	8
	Water	100
Formulation 3	Glycerol	5
	Sodium stearate	3
	Cetostearyl alcohol	2
	Liquid paraffin	25
	Water	65

3.6.2 Efficacy of the topical preparations *in-vitro* on *Staphylococcus aureus*

The formulations were tested with the pour plate well diffusion assay. Bacteria were grown in Mueller-Hinton broth until specific optical density (OD) that gave 1×10^8 cfu/mL. 6 mL of molten Mueller-Hinton soft agar inoculated with 100 μ L of bacteria were poured on Mueller-Hinton agar plate and quickly swirled to get an even distribution of bacteria. 5 mm wells were made with a cork borer and the formulations were dispensed into the well using a Luer-slip 1 mL syringe. Volume of formulation dispensed into each well was 50 μ L. Plates were incubated at 37 °C overnight. The antimicrobial activity was expressed as the mean diameter of the inhibition zone (mm).

3.6.3 Efficacy of Formulation 1 on *Staphylococcus aureus*

The pour plate well diffusion assay was performed as previously described in 3.6.2, with repetitions on day 1, 3, 5, 7 and 10 after the formulation was prepared, to observe the effectiveness of the formulation throughout 10 days. Results were measured as the mean diameter of inhibition in mm.

3.6.4 *In vivo* study of the topical formulation on guinea pigs

The antibacterial property of the formulation was assessed by *in vivo* colonization. Guinea pigs were anesthetized by diethyl ether prior experiment. The back of the guinea pigs were clipped free of fur (2 cm x 2 cm) and swabbed with 70 % ethanol. A cut was made on the skin and infected with the

bacterial inoculum of *Staphylococcus aureus* (50 µL). The infections on the guinea pigs were observed for 2 days. On the 3rd day, the infected areas were administered with the formulation containing the active fraction, placebo formulation, a positive standard (Burnol[®]) and one patch are left untreated (six guinea pigs per group). Treatments lasted for 10 days and data were collected on the 11th day. The number of viable organisms was determined by the swabbing method as described by Rubinchik et al. (2009). Colony counts were determined by swabbing the infected area with a sterile cotton-tip. The sampling solutions were then further diluted and plated for colony counts (Rubinchik et al., 2009).

3.7 Synergistic Studies of the Active Fraction F5 with Antibiotics

3.7.1 Checkerboard assay

To evaluate presence of synergism or antagonism of the samples with antibiotics, isobolograph analyses were done applying the checkerboard assay with reference to White et al., (1996) and Tallarida (2001). This method involves varying the concentrations of the samples and the antibiotics along different axes ensuring that each well contained different combinations of the samples and the antibiotics as in Figure 3.1.

The antibiotics used were Ampicillin, Tetracycline and Streptomycin. The analyses were performed in 96 well plates. Bacteria were grown to reach optical density as of 2×10^8 cfu/mL. 5 microliters of the inoculums were added into the well containing samples, antibiotics and MHB. Total volume in each well was 200 μ L.

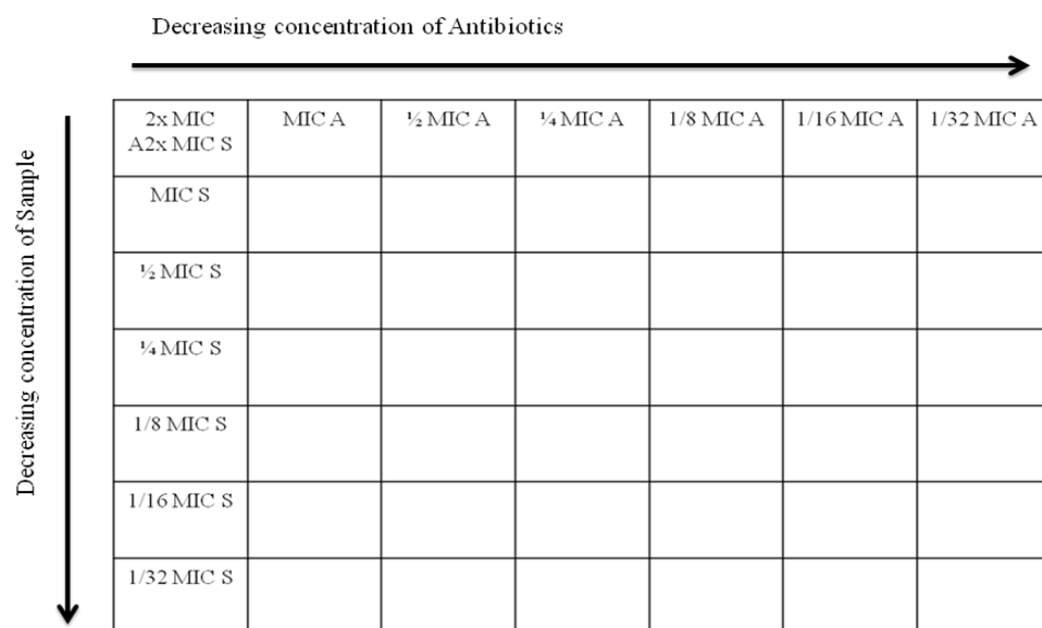


Figure 3.1. Layout of the 96-well plate for the execution of the Checkerboard assay

The plates were incubated for 18 hours at 37 °C. MIC of the combination was determined as the lowest concentration which completely inhibited bacterial growth. To evaluate the effect of the combinations, fractional inhibitory concentration (FIC) were calculated for each combination using the following formula:

$$FIC_A = MIC_A \text{ in combination} / MIC_A \text{ alone}$$

$$FIC_B = MIC_B \text{ in combination} / MIC_B \text{ alone}$$

$$FIC \text{ index} = FIC_A + FIC_B$$

FIC_A is the FIC of the antibiotic, MIC_A is the MIC of the antibiotic, FIC_B is the FIC of the sample and MIC_B is the MIC of the sample. FIC index is the FIC added value of both the antibiotic and the samples. The interaction of the antimicrobial combinations was determined by plotting an isobologram as previously reported by Mandalari et al. (2010) and Jayaraman et al. (2010).

3.7.2 Time-kill assay

The time-kill method to analyze synergism follows the same procedure in the microbroth dilution of the MIC determination. Different combinations of samples and antibiotics were added into different wells. The optical density of the plates was observed at 600 nm with a microplate reader every 1 hr for 18 hrs. The absorbance were plotted to define the growth curve of each combination. The curves of the sample alone, antibiotics alone and the

combination were compared to assess viable colony counts against time (Tallarida 2001).

3.8 FESEM Analysis on Cell Membrane Integrity of *Staphylococcus aureus* and Methicilin resistant *Staphylococcus aureus* (MRSA)

FESEM analysis was conducted to observe the morphology of bacteria cells upon treatment with the ethanol extract and F5. A high resolution FESEM was used (FEI Quanta 400). Untreated bacteria cells were used as controls. *Staphylococcus aureus* and Methicilin resistant *Staphylococcus aureus* were cultured overnight in MHB at 37 °C. Cells were treated with the MIC dose of the samples, and left again overnight. It is then harvested by centrifugation at 5000 rpm for 10 mins. Cells were washed twice with 0.1M phosphate buffer (pH 7.2) and fixation was done by immersing the cells in 2.5 % formaldehyde overnight at 4 °C. After fixation, cells were washed again with phosphate buffer twice before serially dehydrated with increasing ethanol water mixtures (30 % - 100 %) for 10 minutes each gradient. Cells were left in 100 % ethanol to mount on the metal stubs for viewing.

CHAPTER 4

RESULTS

4.1 Plant Collection and Extraction

Acalypha wilkesiana var. *macafeana* hort. was collected and authenticated by the Forest Research Institute Malaysia (FRIM). Voucher specimens were pressed and deposited at the School of Pharmacy, The University of Nottingham Malaysia Campus (UNMC 9W). The percentage yields of extracts are expressed as per dry weight of plant material (Table 4.1). The highest percentage yield was obtained from extraction with water, while the least is from ethyl acetate. Percentage yield was calculated per dry weight of the grinded plant material.

4.2 Fractionation of the Ethanol Extract

The ethanol extract was chosen to be separated further via chromatography as it was the active extract for both antioxidant and antibacterial screening. The ethanol extract was separated through Sephadex LH-20 column and 5 fractions were afforded with the yield of the fractions as in Table 4.2. The fractionation progress was captured in Figure 4.1. Percentage yield was calculated as per dry

ethanol extract used for fractionation. All fractions were obtained as solid materials except for F1 which was in sticky liquid form. The highest yield was obtained from fractionation with 25 % aqueous ethanol.

4.3 Antioxidant Properties of Fractions

Qualitative assessment with DPPH spray on the TLC chromatographic profiles of the fractions showed long tailing bands of yellow shade, which did not specifically show the antioxidant active compound or fraction as pictured in Figure 4.2. This emphasizes the importance for evaluation to be done quantitatively. To evaluate quantitatively the antioxidant potential of the ethanol extract of *A. wilkesiana* var. *macafeana* hort. and its' fractions, three antioxidant tests were performed i.e., FRAP assay, DPPH radical scavenging assay and β -carotene bleaching assay. Results are presented in Table 4.3. The total phenolic content of the samples was also assessed with Folin-Ciocalteu reagent, and the results are presented in Figure 4.3.

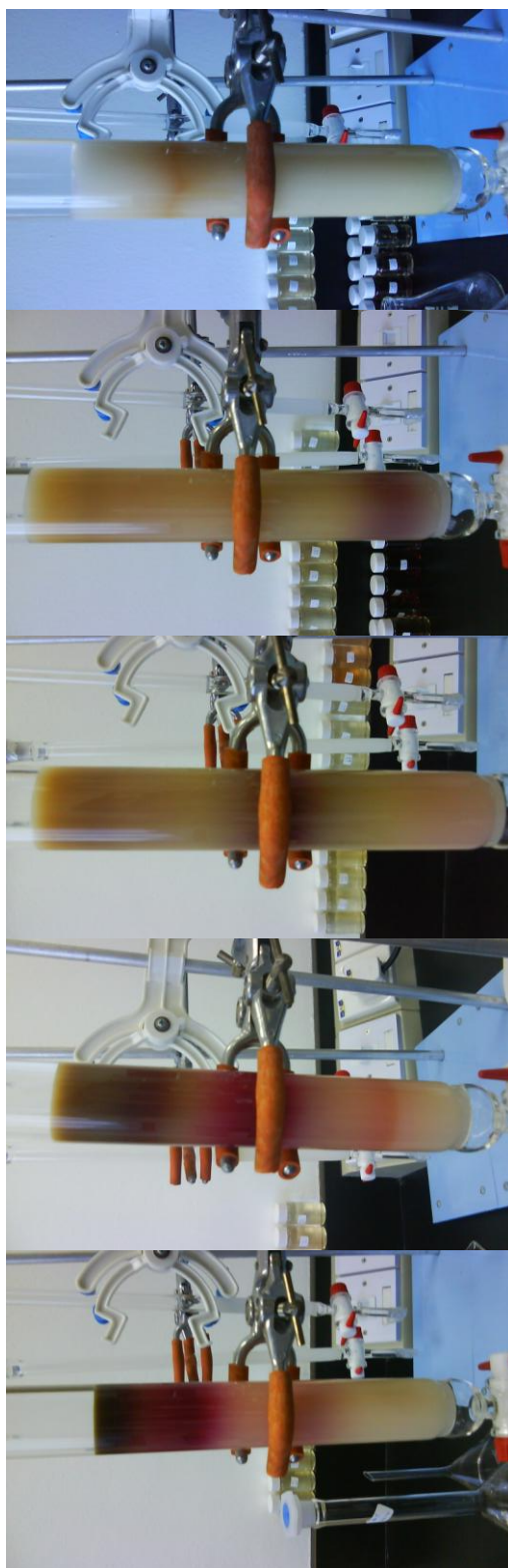


Figure 4.1. Process of fractionation with Sephadex LH-20. (Picture from left to right).

Table 4.1 Yield of extraction and form of each extract.

Extract	Yield (%)	Form
Hexane	0.32	Sticky dark brown liquid
Ethyl acetate	0.18	Sticky dark green liquid
Ethanol	3.29	Dark red solid
Water	15.84	Dark brown solid

Table 4.2 Yield of fractionation and form of each fraction.

Fraction	Form	Solvent	Yield (%)
F1	Dark yellow sticky liquid	25 % aq. Ethanol	18.9
F2	Dark red solid	50 % aq. Ethanol	17.6
F3	Dark purple solid	75 % aq. Ethanol	12.4
F4	Dark purple solid	100 % Ethanol	15.2
F5	Dark brown/golden solid	70 % aq. Acetone	17.9

Interestingly, our research showed that F5 displayed the most prominent antioxidant effect in all three assays as compared to other fractions and the crude ethanol extract with antioxidant capacity of $2.090 \pm 0.307 \mu\text{g/mL}$, $0.532 \pm 0.041 \mu\text{g/mL}$, $0.032 \pm 0.025 \mu\text{g/mL}$ in FRAP, DPPH and BCB assay, respectively. Additionally, F5 was more active than positive standards used in the three assays i.e., quercetin, Trolox and ascorbic acid. The total phenolic content for F1 and F5 were significantly higher than the ethanol extract with values equal to 208.7 ± 7.02 , 179.57 ± 17.96 g of gallic acid equivalent/100 g sample, respectively. Clear correlation is seen between the antioxidant potentials and the total phenolic content, thus suggesting that the phenolics account for antioxidant activity of the plant.

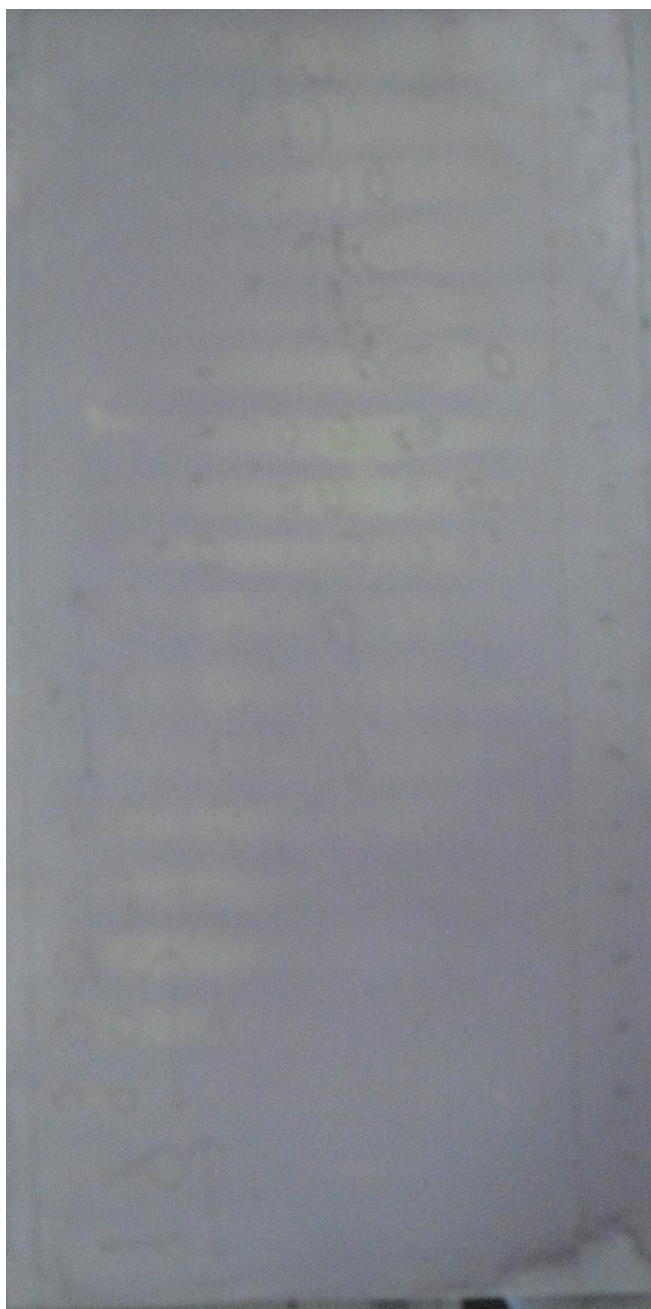


Figure 4.2 Thin Layer Chromatography (TLC) plates sprayed with DPPH spray which results in yellow tailing bands of antioxidant bioactives in purple background.

Table 4. 3 Antioxidant properties of samples evaluated with FRAP, DPPH and BCB assay.

Sample	FRAP	DPPH	BCB
	FE ($\mu\text{g/mL}$)	IC ₅₀ ($\mu\text{g/mL}$)	EC ₅₀ ($\mu\text{g/mL}$)
	Mean \pm SEM		
Crude Ethanol	10.613 \pm 2.643	1.172 \pm 0.834	0.122 \pm 0.039
F1	2.660 \pm 0.510	1.048 \pm 0.085	0.082 \pm 0.026
F2	7.604 \pm 0.113	1.222 \pm 0.183	0.194 \pm 0.132
F3	6.670 \pm 0.383	0.902 \pm 0.291	0.401 \pm 0.445
F4	5.018 \pm 0.788	1.482 \pm 0.139	0.089 \pm 0.086
F5	2.090 \pm 0.307	0.532 \pm 0.041	0.032 \pm 0.025
Trolox	8.422 \pm 0.897	Nd	0.120 \pm 0.081
Quercetin	3.072 \pm 0.436	Nd	0.266 \pm 0.177
Ascorbic acid	Nd	1.181 \pm 0.223	Nd

*Nd: Not determined.

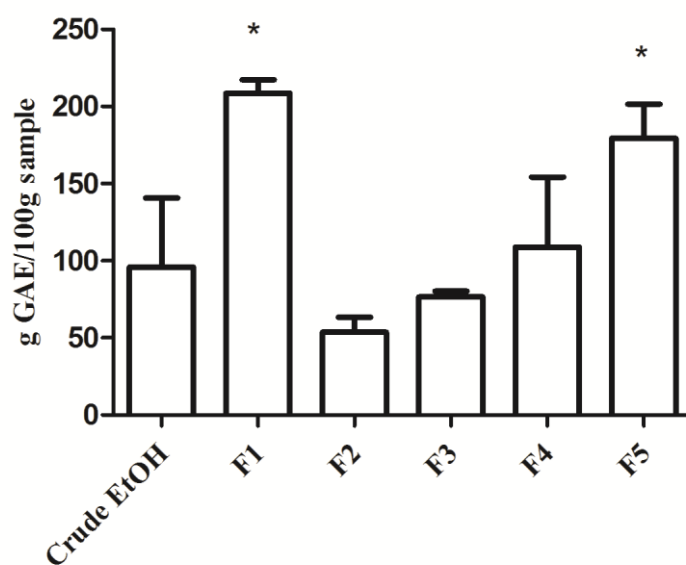


Figure 4.3. Total phenolic content of the fractions. Each bar represents mean \pm SEM of three independent experiments. (* shows significance at $p < 0.05$ vs. ethanol extract).

4.4 Antibacterial Properties of Fractions

The hexane, ethyl acetate and ethanol extracts (100 μg) of *A. wilkesiana* var. *macafeana* hort. were tested against gram-positive *Bacillus cereus* (ATCC 11778), *Bacillus subtilis* (ATCC 6633), *Staphylococcus aureus* (ATCC 11632) and Methicillin resistant *Staphylococcus aureus* (MRSA) clinical strain and gram-negative *Escherichia coli* (ATCC 8739), *Pseudomonas aeruginosa* (ATCC 10145), and *Citrobacter freundii* (ATCC 8090). As shown in Table 4.4, noticeable antibacterial activities were observed with the ethyl acetate and ethanol extract whereby the hexane extract was inactive. The inhibition zone diameters of the ethyl acetate and ethanol extracts were equal to 8.06 ± 0.32 mm and 9.36 ± 1.22 mm against *B. subtilis* (ATCC 6633), and 8.42 ± 0.58 mm and 8.73 ± 0.92 mm against *S. aureus* (ATCC 11632), respectively.

Interestingly, the ethyl acetate and ethanol extract (100 μg) inhibited the growth of MRSA with inhibition diameters equal to 7.20 ± 0.94 and 7.31 ± 0.38 mm, respectively. Both antibiotics used at a dose of 5 μg were inactive against MRSA. Both ethyl acetate and ethanol extract incurred partial inhibition zones against gram-negative *E. coli* (ATCC 8739) with inhibition diameters equal to 8.13 ± 0.94 mm and 13.48 ± 0.10 mm, respectively. Partial inhibition is noted if there are resistant colonies growing on the inhibition

zones. The ethanol extract expressed the highest antibacterial activity and account for the antibacterial properties of *A. wilkesiana* var. *macafeana* hort.

To further locate the active constituents, the ethanol extract was passed through a Sephadex LH-20 column affording 5 fractions. The antibacterial potencies of the 5 fractions were tested with the pour plate disc diffusion assay against gram-positive *Bacillus cereus* (ATCC 11778), *Bacillus subtilis* (ATCC 6633), *Staphylococcus aureus* (ATCC 11632) and Methicillin-resistant *Staphylococcus aureus* (MRSA) clinical strain and gram-negative *Escherichia coli* (ATCC 8739), *Pseudomonas aeruginosa* (ATCC 10145), and *Citrobacter freundii* (ATCC 8090) (Table 4.5).

After the incubation, the highest antibacterial activity was elicited by the fifth fraction (F5) with inhibition zones equal to 11.01 ± 1.53 mm, 16.63 ± 0.11 mm, 11.40 ± 1.10 mm and 8.22 ± 0.19 mm in *Bacillus cereus* (ATCC 11778), *Bacillus subtilis* (ATCC 6633), *Staphylococcus aureus* (ATCC 11632) and MRSA (clinical strain) respectively. Ampicillin and streptomycin were inactive at a dose of 5 μ g against MRSA whereas F5 was active against all the gram-positive bacteria. F5 is the last fraction eluted with aqueous acetone from Sephadex LH-20 column implies the fact that it consists of high molecular weight molecule (Hagerman 2002).

Table 4.4 Diameter inhibition of extracts.

Sample	Inhibition diameter (mm, SD)						
	Gram positive				Gram negative (partial inhibition)		
	<i>B. cereus</i>	<i>B. subtilis</i>	<i>S. aureus</i>	MRSA	<i>E. coli</i>	<i>P. aeruginosa</i>	<i>C. freundii</i>
Hexane	NI	NI	NI	NI	NI	NI	NI
Ethyl Acetate	8.06 ± 0.32	8.14 ± 0.42	8.42 ± 0.58	7.20 ± 0.26	8.13 ± 0.94	NI	9.56 ± 1.20
Ethanol	9.36 ± 1.22	14.22 ± 0.13	8.73 ± 0.92	7.31 ± 0.38	13.48 ± 0.10	NI	12.82 ± 3.52
Ampicillin	8.12 ± 1.26	NI	15.19 ± 0.62	NI	11.95 ± 0.70	NI	NI
Streptomycin	12.31 ± 1.64	13.77 ± 0.48	10.44 ± 0.40	NI	10.84 ± 0.51	NI	14.32 ± 1.47
DMSO	NI	NI	NI	NI	NI	NI	NI

* NI - no inhibition observed. Doses of the samples were 100 µg per disc while for antibiotics were 5 µg per disc.

Table 4.5 Inhibition diameters of fractions from the ethanol extract.

Sample	Inhibition diameter (mm, SD)							
	Gram positive				Gram negative (partial inhibition)			
	<i>B. cereus</i>	<i>B. subtilis</i>	<i>S. aureus</i>	<i>MRSA</i>	<i>E. coli</i>	<i>P. aeruginosa</i>	<i>C. freundii</i>	
Crude Extract	9.36 ± 1.22	14.22 ± 0.13	8.73 ± 0.92	7.31 ± 0.38	13.48 ± 0.10	NI	12.82 ± 3.52	
F1	NI	NI	NI	NI	NI	NI	NI	
F2	6.68 ± 0.63	NI	NI	NI	NI	NI	NI	
F3	7.18 ± 0.71	14.52 ± 0.28	7.30 ± 0.15	NI	NI	NI	NI	
F4	10.47 ± 0.85	16.40 ± 0.01	9.60 ± 1.10	NI	14.83 ± 0.04	NI	NI	
F5	11.01 ± 1.53	16.63 ± 0.11	11.40 ± 1.10	8.22 ± 0.19	15.28 ± 0.70	9.98 ± 2.46	13.74 ± 3.52	
Ampicillin	8.12 ± 1.26	NI	15.19 ± 0.62	NI	11.95 ± 0.70	NI	NI	
Streptomycin	12.31 ± 1.64	13.77 ± 0.48	10.44 ± 0.40	NI	10.84 ± 0.51	NI	14.32 ± 1.47	
DMSO	NI	NI	NI	NI	NI	NI	NI	

* NI - no inhibition observed. Doses of the samples were 100 µg per disc while for antibiotics were 5 µg per disc.

We raised the question of whether F5 treatment affects the survival or division of bacteria and therefore determined the minimum inhibitory concentration (MIC) of F5 and observed that F5 inhibited bacterial multiplication of MRSA at concentration of 750 $\mu\text{g/mL}$. Bactericidal effects against *S. aureus* (ATCC 11632) and MRSA were evidenced with the minimum bactericidal concentration (MBC) assay which yielded a MBC value equal to 3 mg/mL (Table 4.6).

4.6 Identification of Constituents in F5

The identity of the compounds in F5 was identified by fragmentations of the compounds via Bruker MicroTOF II MS system. Sample was dissolved with 20 % acetonitrile and separation of the constituents was performed on a Zorbax C_{18} column (4.6 mm x 150 mm, 5 μm particle size) from Agilent Technologies with gradient acetonitrile and water mixtures. The peaks were detected at 280 nm as this wavelength is the wavelength for detection of tannins. All the peaks were then diverted to the MS system and scanned from m/z 100 – 1500 for detection of the fragments. All MS analysis was performed in negative ionization. HPLC profiling of the active fraction F5 were optimized in analytical scale and were performed before any MS analysis were done. HPLC chromatographic profile is captured in Figure 4.4.

Table 4.6. MIC and MBC

Sample	MIC ($\mu\text{g/mL}$)				MBC ($\mu\text{g/mL}$)			
	<i>S. aureus</i>	<i>B. cereus</i>	MRSA		<i>S. aureus</i>	<i>B. cereus</i>	MRSA	
Crude extract	1500	3000	1500		3000	>3000	3000	
F5	750	3000	750		3000	>3000	3000	
Ampicillin	50	3.125	> 100		50	3.125	>200	
Streptomycin	1.563	0.391	100		1.563	0.391	>100	
Tetracycline	0.625	Nd	> 20		0.625	Nd	> 20	

* Nd: Not determined

To gain further insights to the nature of active constituents of F5 liquid chromatography/mass-spectrometry (LC/MS) analysis was performed allowing the identification of a major tannin. The major tannin (**2**) was identified as geraniin (54.9 %) in line with evidence provided by Self et al. (1986). Percentage of other compounds in F5 was in the range of 0.2 % to 5 %. The resulting chromatogram is shown in Figure 4.4. Geraniin forms an equilibrium mixture of a hydrated five-membered hemiacetal ring structure and a hydrated six-membered hemiacetal-ring upon mutarotation (Okuda et al., 1982).

Other compounds present in F5 were also analyzed using their fragmentation patterns as tabulated in Table 4.7 and referenced to the literature. LC-MS/MS method was needed to distinguish between presence of quercetin or ellagic acid as both compounds produce molecular ions of $[M-H]$ m/z 301 (Mullen et al., 2003). MS/MS analysis showed that further fragmentation of m/z 301 yielded characteristic daughter ions of m/z 257 and 229 which is the unique pattern for ellagic acid (Seeram et al., 2006). All of the peaks except peak (**1**) and (**7**), yielded a fragment of m/z 301, and showed the same daughter ion fragments, thus confirming that the peaks are ellagitannins.

Peak (**1**) is β -D-glucogalline which yields ion fragments of m/z 331 and 169 (Figure 4.7) with the same respective fragments identified by Pawlowska et al.

(2010). The fragments that we propose to justify the fragmentation are shown in Figure 4.8. β -D-glucogalline is a simple gallotannin which comprises of gallic acid and a β -D-glucose. Peak (4) fragments were referenced to Hager et al. (2008) as fragmentation ions yield upon MS were m/z 935 and m/z 301 (Figure 4.9) and was identified as Potentillin. The fragment at m/z 301 is ellagic acid. The fragmentation proposed to justify for the fragmentations are represented in Figure 4.10. Peak (7) showed a m/z 935.0 (Figure 4.11) which was shown to be double charged $[M-2H]^{2-}$ by zoom scan analysis giving its real m/z as 1870 for the peak as referenced in Hager et al. (2008) as sanguin H-6 or lambertianin A. The zoom scan analysis spectrum is shown as Figure 4.12.

The major compound geraniin (2) was then isolated and freeze dried before analysis with NMR to elucidate. Samples were able to be isolated in the pure form enough for only NMR analysis. Further experiments with the pure form of geraniin were not able to be executed due to the quantity of the pure Geraniin isolated. NMR analysis interpreted as shift of ^1H NMR and ^{13}C NMR are tabulated in Table 4.8 and Table 4.9 with comparison to existing literature. The comparison shows that the identification of the compound is geraniin, in correspondence with findings by LC-MS. Geraniin forms an equilibrium mixture of 2a and 2b in an aqueous solution (Okuda et al., 1982). Upon

equilibration, each carbon shows two peaks in the ^{13}C NMR spectra at a ratio of 1:1 (Appendix).

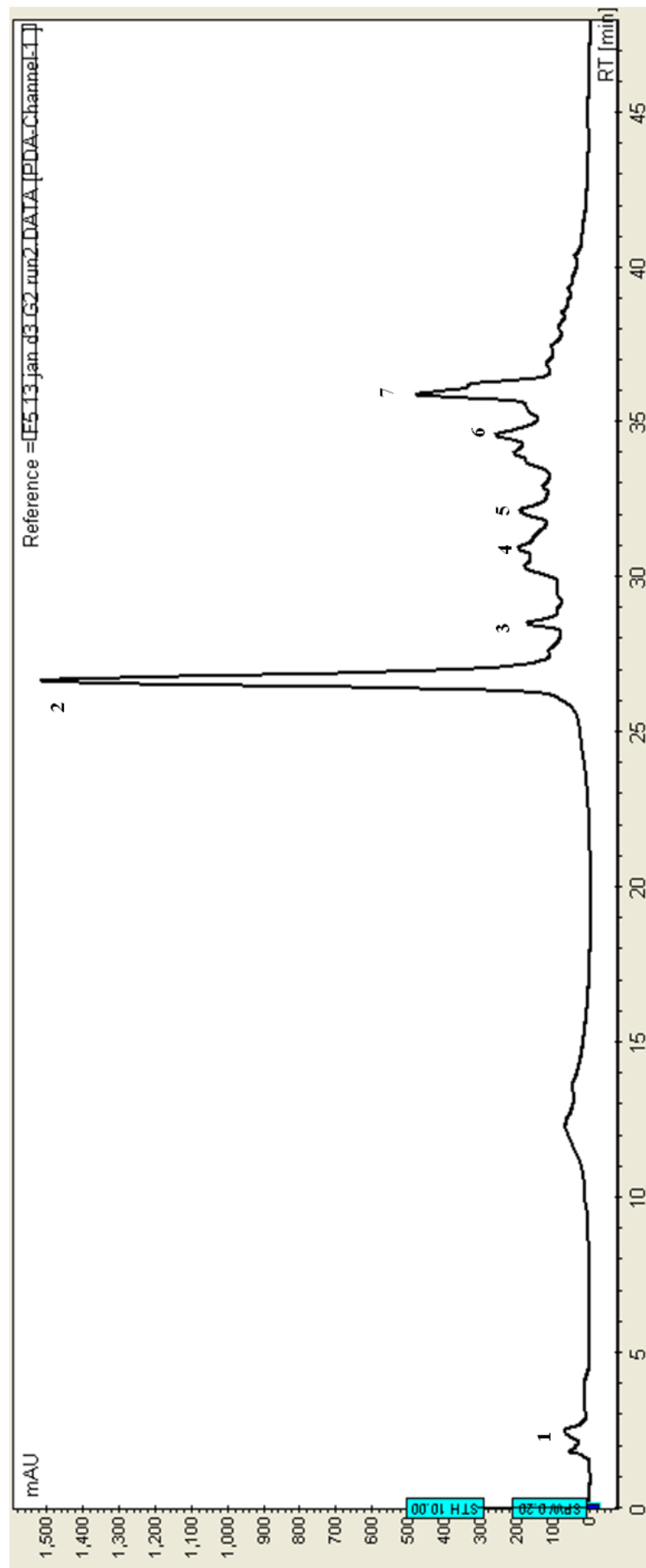


Figure 4.4. Chromatographic profile of F5. (HPLC)

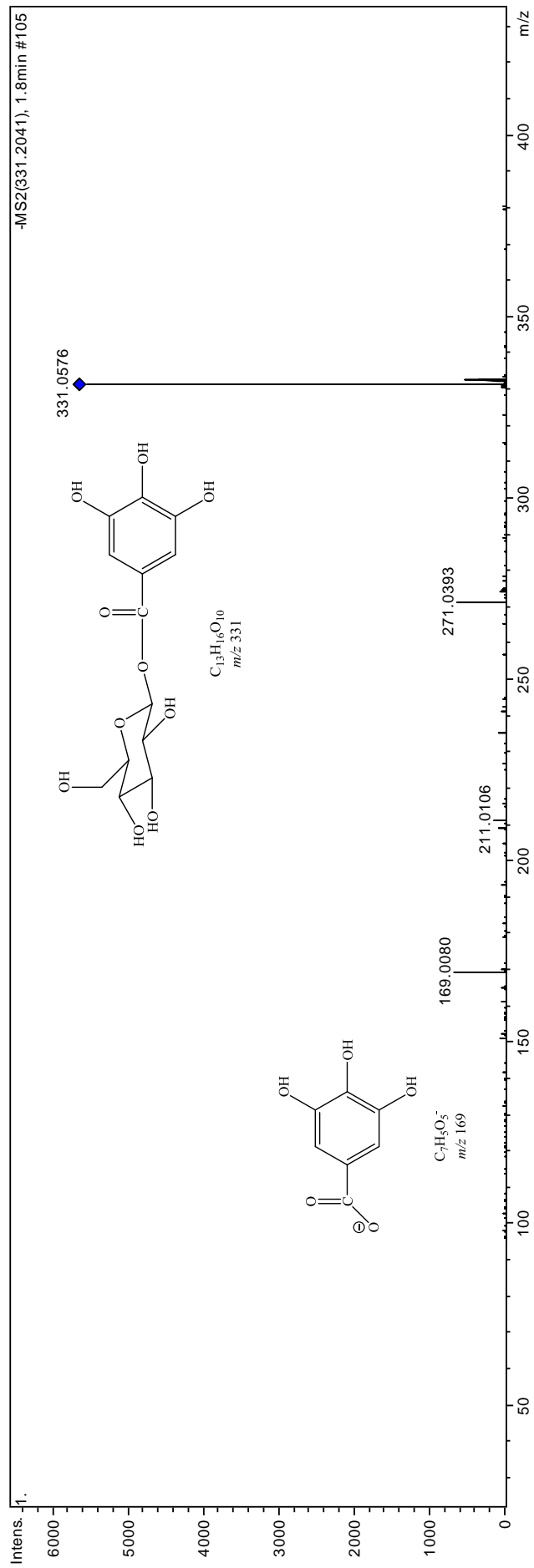


Figure 4.5. LC-MS spectra of Compound 1

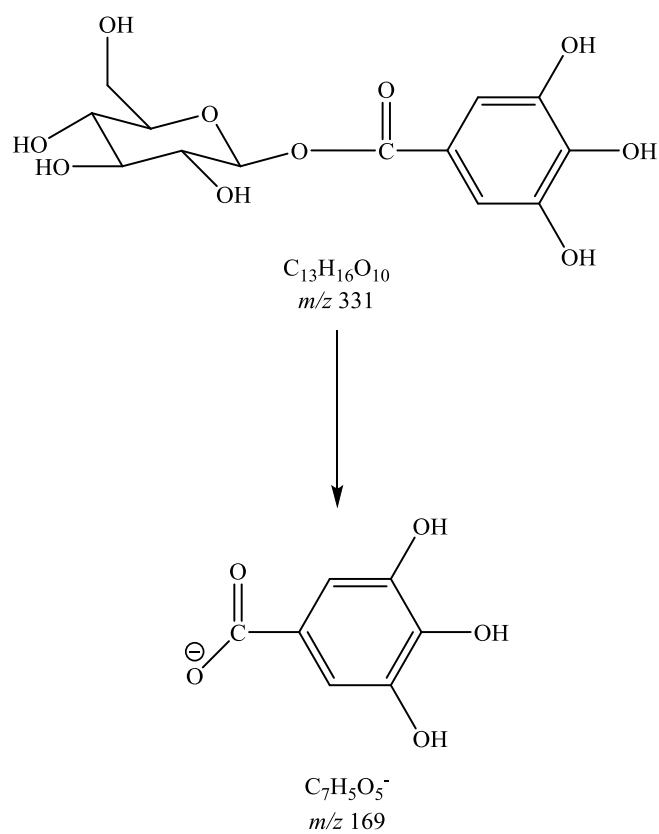


Figure 4.6. Fragments proposed to justify fragmentation observed in mass spectrum of compound 1 (β -D-glucogalline).

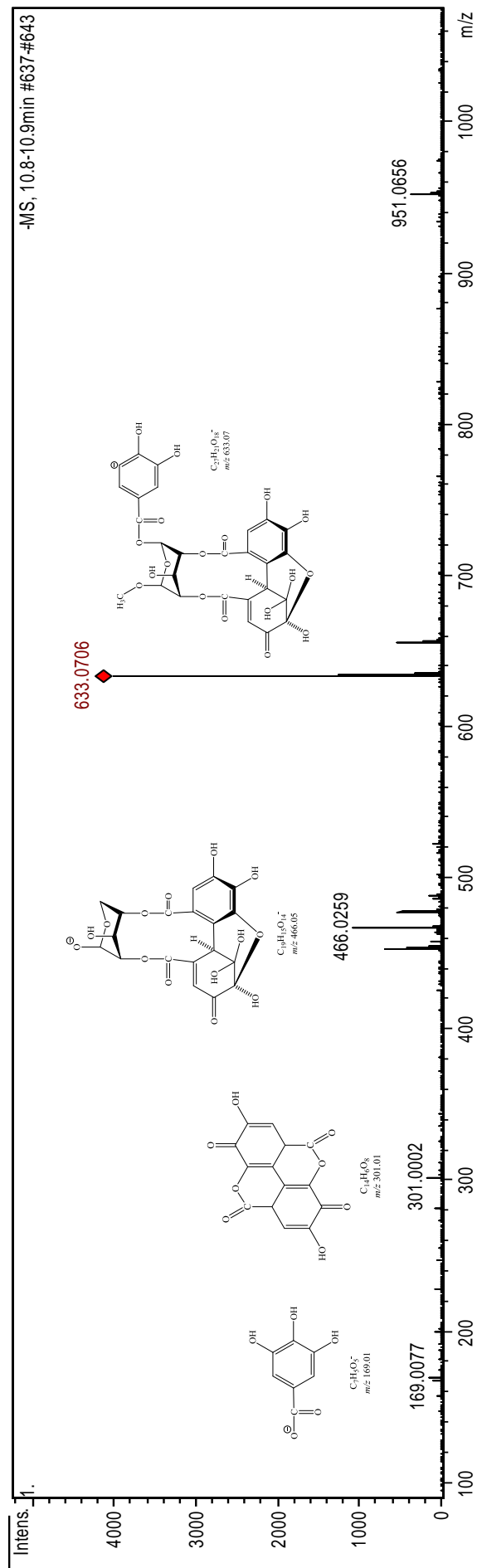
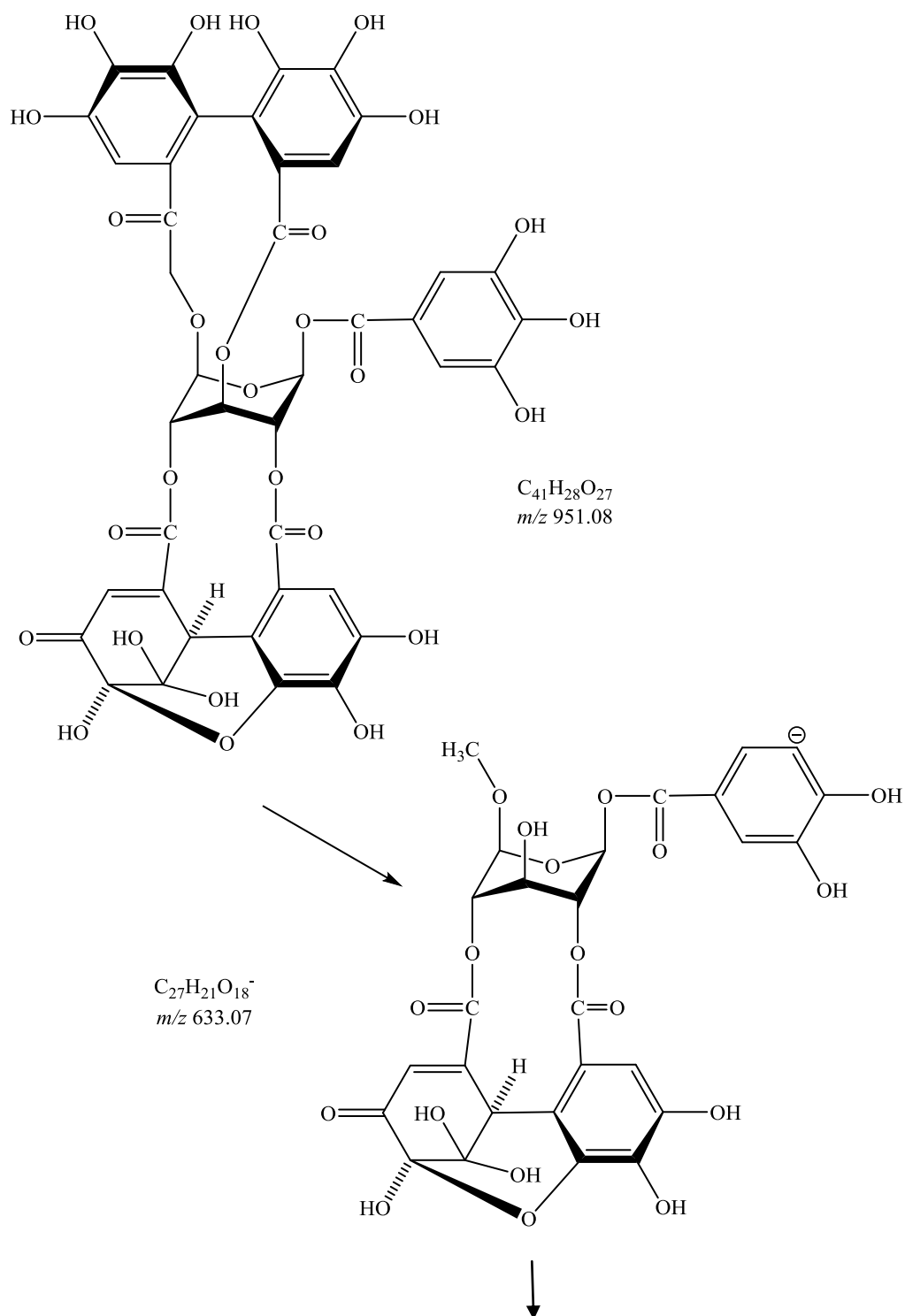


Figure 4.7. LC-MS spectra of Compound 2



(Continued)

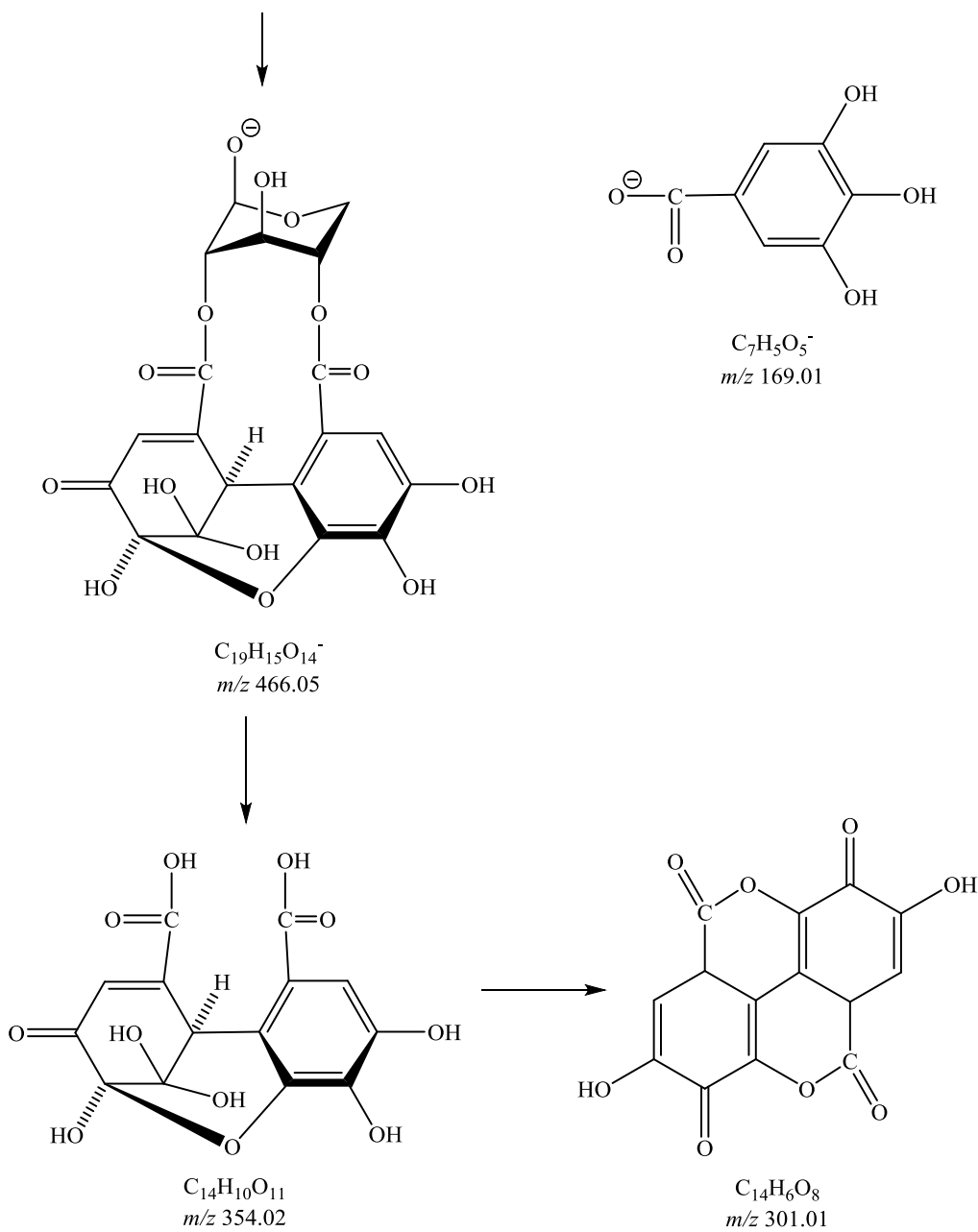


Figure 4.8. Fragments proposed to justify fragmentation observed in mass spectrum of compound 2 (Geraniin).

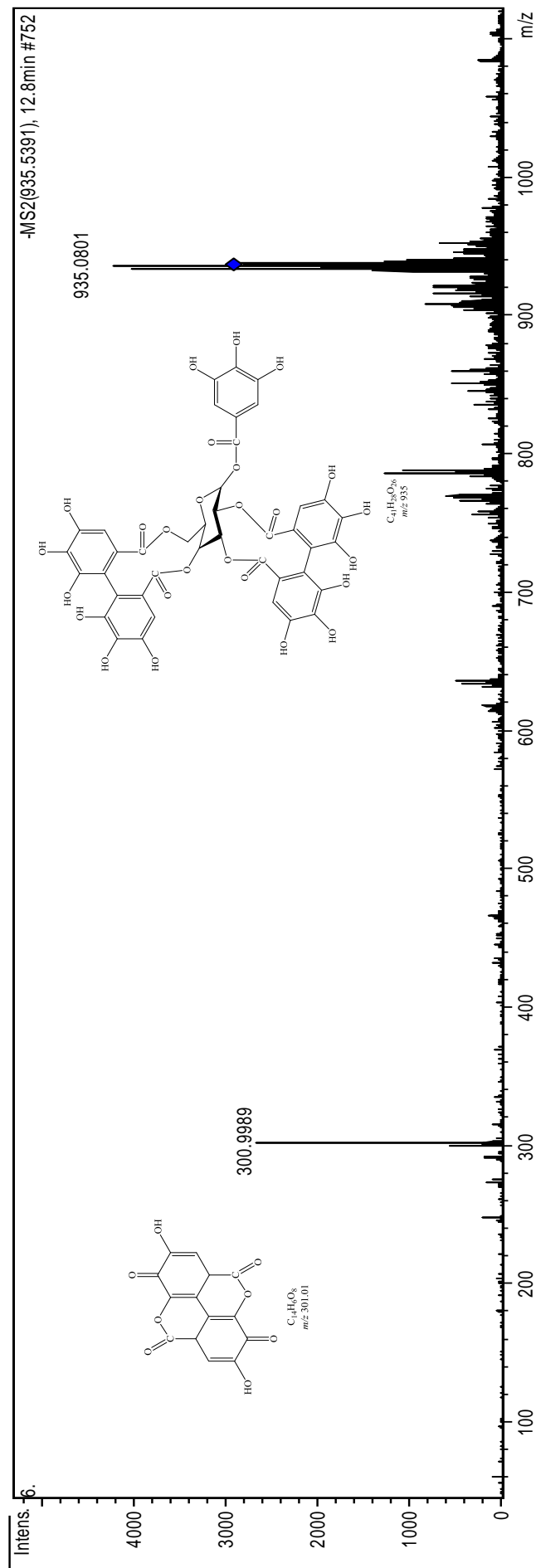


Figure 4.9. LC-MS spectra of Compound 4

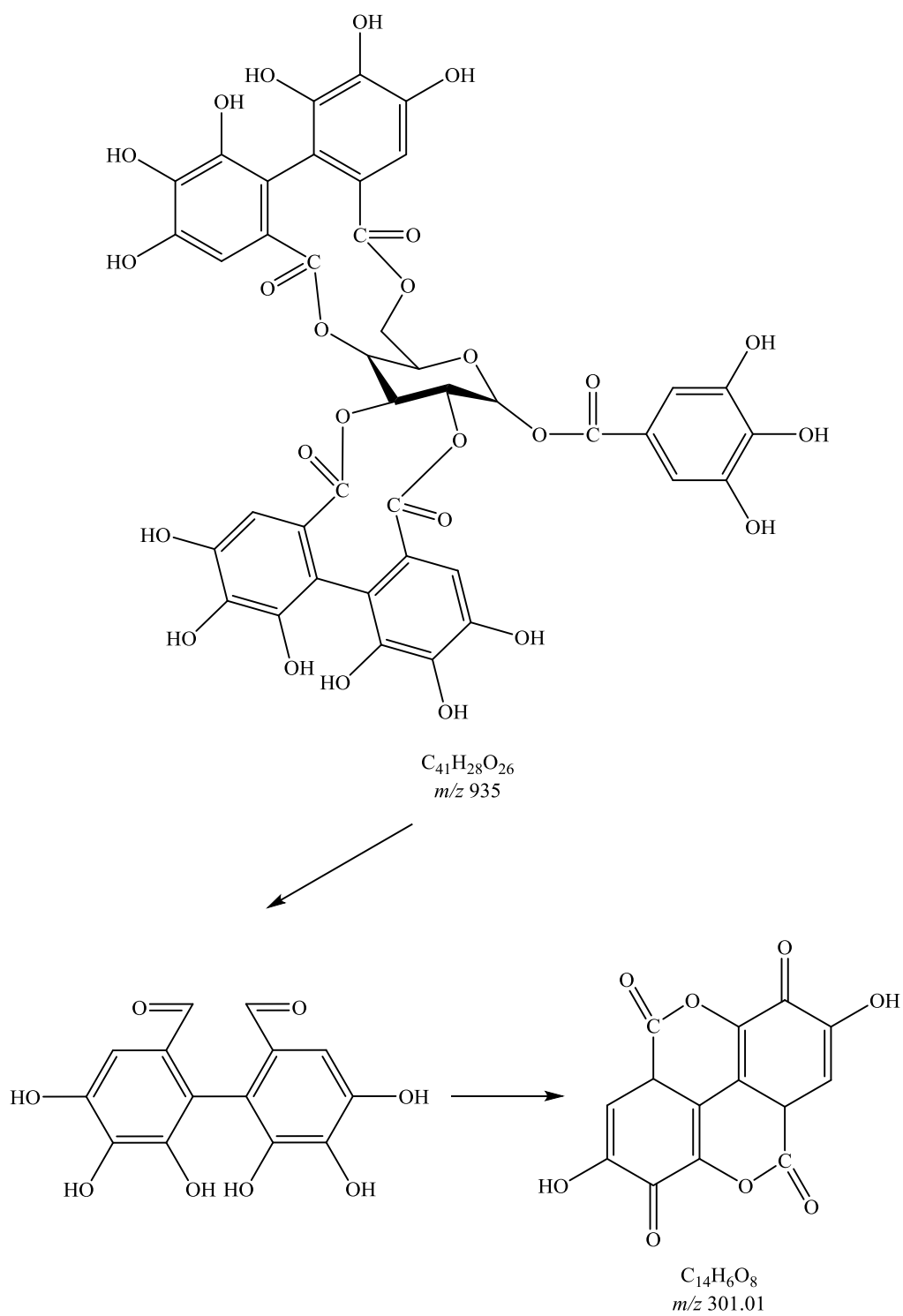


Figure 4.10. Fragments proposed to justify fragmentation observed in mass spectrum of Compound 4 (Potentillin).

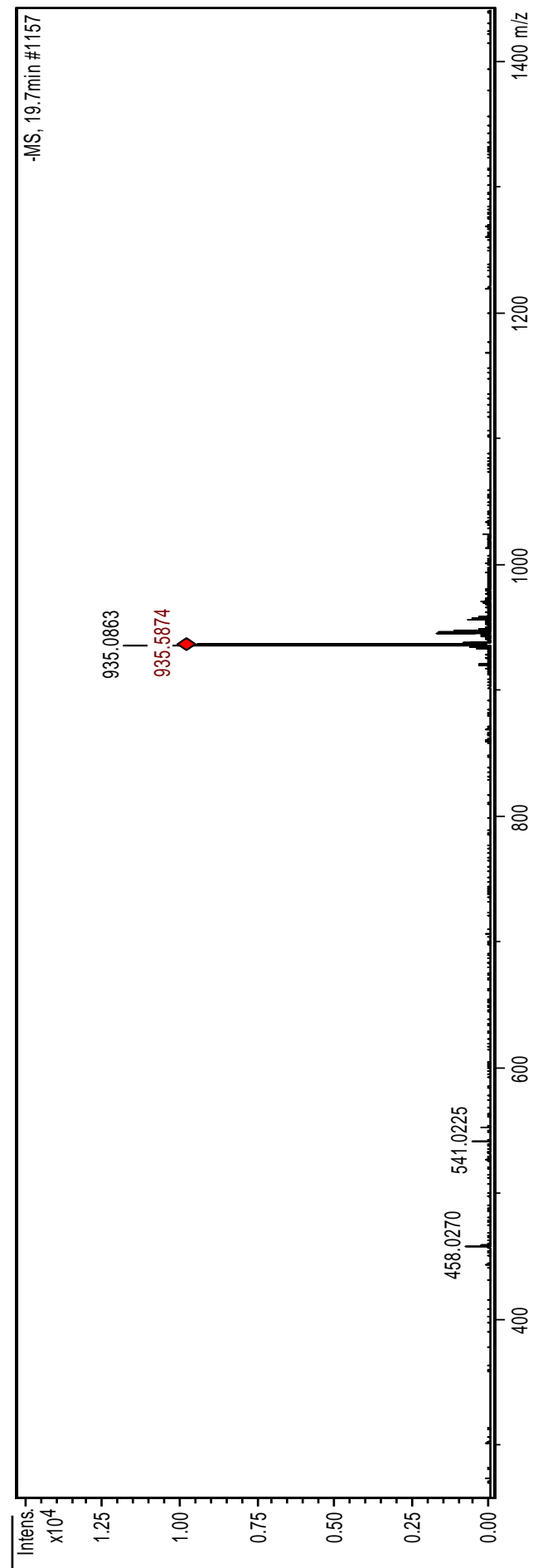


Figure 4.11. LC-MS spectra of Compound 7

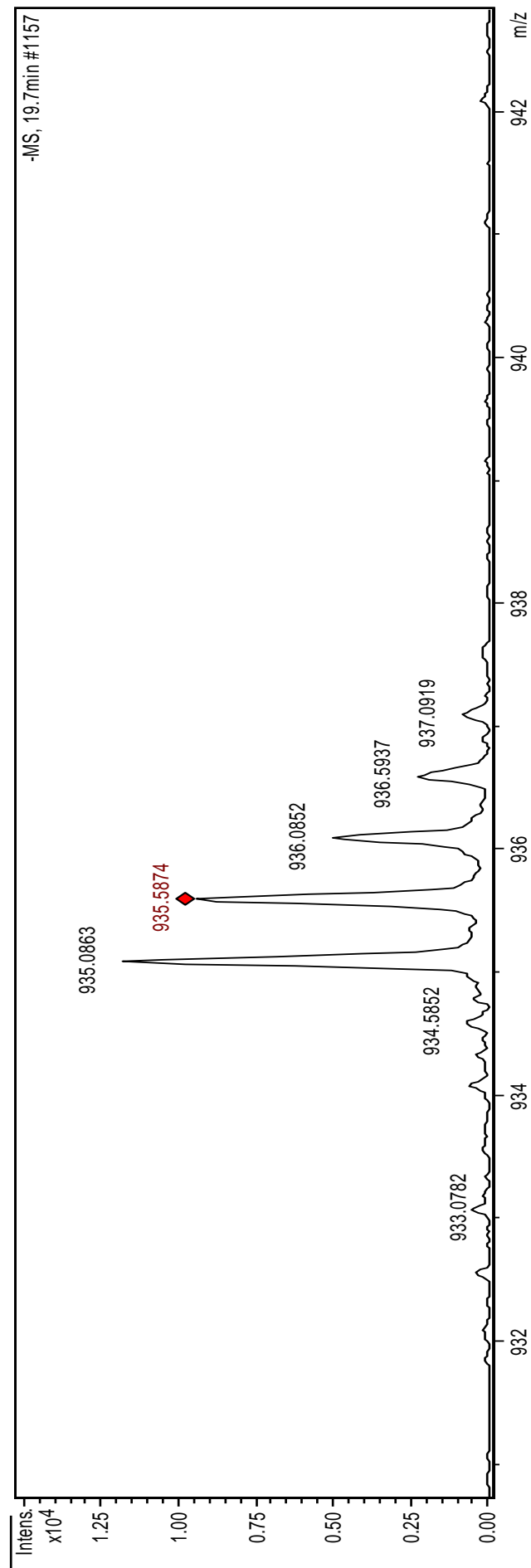
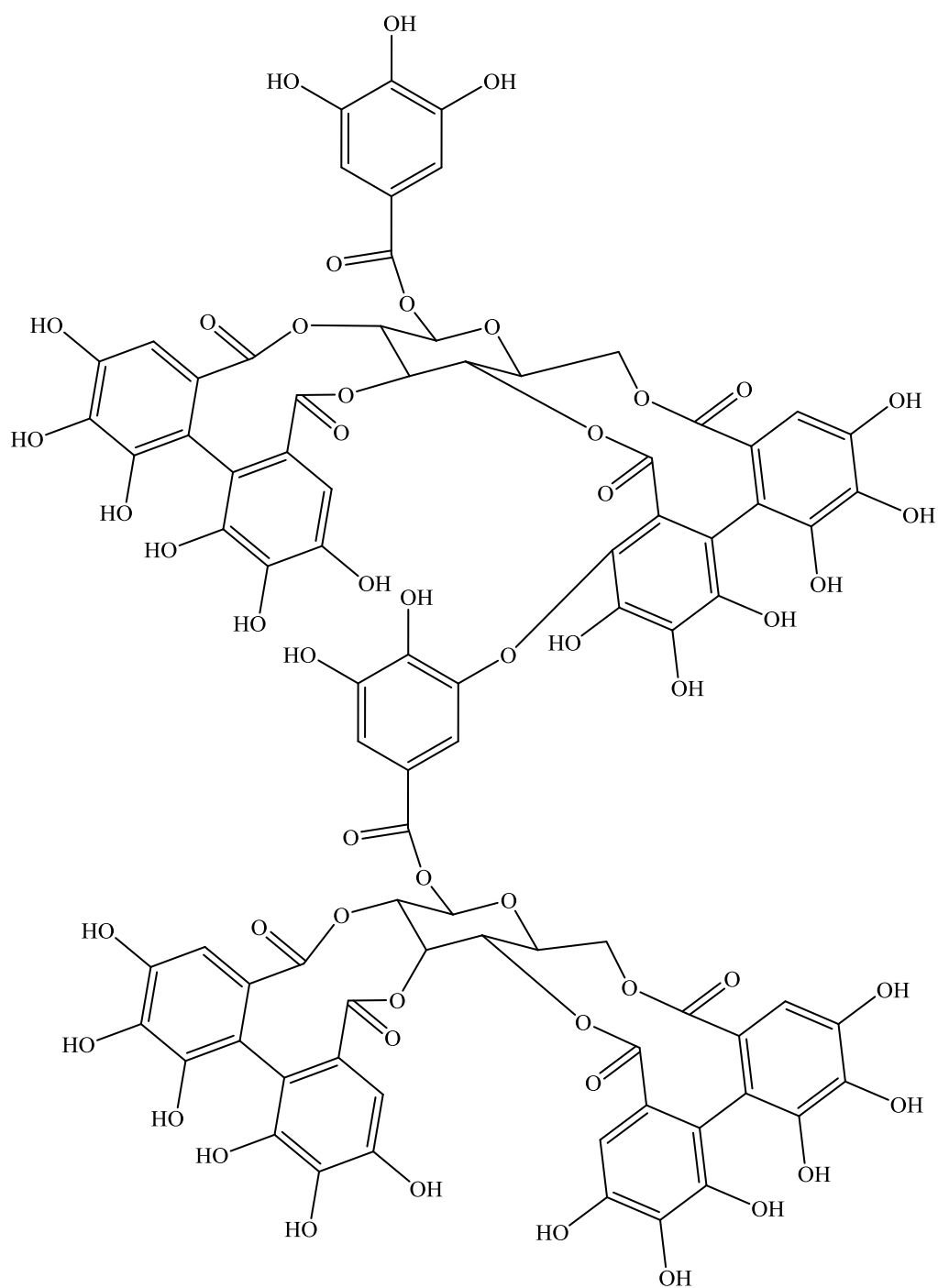


Figure 4.12. Doubly charged peak at m/z 935 (zoom scan analysis)



C₈₂H₅₄O₅₂
m/z 1870.16



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(Continued)

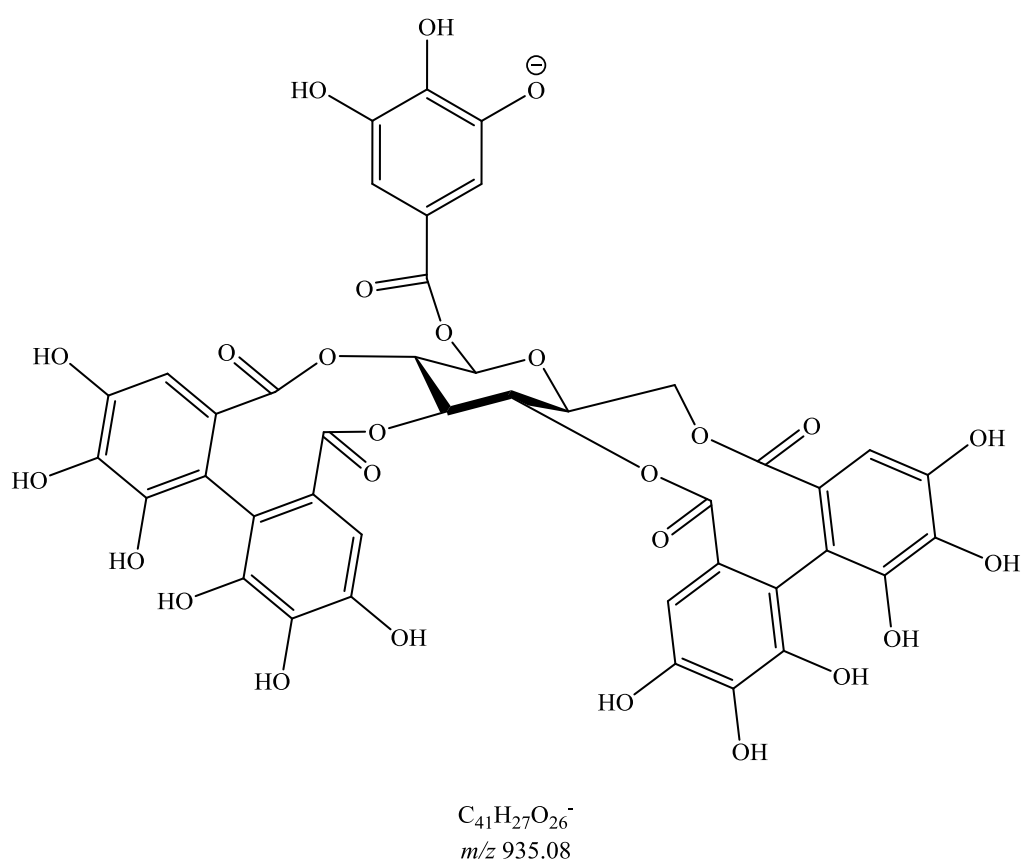


Figure 4.13. Fragments proposed to justify fragmentation observed in mass spectrum Compound 7 (Sanguin H-6)

Table 4.7. Summary of fragments identified by LC-MS.

No.	R _t (min)	%	<i>m/z</i>	MS/MS breakdown	Compound	Ref.
1	1.8	0.2	331.1	169.0	β-glucogallin	Pawlowska et al., 2006
2	26.5	54.9	951.1	633.1, 466.0, 301.0, 169.0	Geraniin	Self et al., 2010
3	28.5	3.0	1109.1	933.1, 554.0, 467.0, 301.0, 175.0	Unidentified ellagitannin	None
4	31.0	5.9	935.1	301.0	Potentillin	Hager et al., 2008
5	32.0	3.7	1890.1	935.1, 769.1, 301.0	Unidentified ellagitannin	None
6	34.8	4.2	1890.1	935.1, 769.1, 633.1, 301.0	Unidentified ellagitannin	None
7	36.0	4.2	935 (doubly charged) 1870.2	541.0, 458.0	Sanguin H-6	Clifford & Scalbert, 2000

Table 4.8. ¹H-NMR data of compound **2** in acetone-*d*₆ plus D₂O (referenced relative to solvent peak = 2.05 ppm) with comparison to existing literature (Appendix: NMR spectra of compound **2**).

Moiety	H	NMR shifts			
		1 a (Ref ¹)	Current work	1b (Ref ²)	Current work
Glucose	H-1	6.17 (dd, <i>J</i> = 1.2, 1.6)	6.55 (t <i>J</i> = ca. 1.1)		6.53 (t <i>J</i> = ca. 1.1)
	H-2	5.57 (dt, <i>J</i> = 1.2, 2.4)	5.55 (m)		5.54 (m)
	H-3	5.48 (ddd, <i>J</i> = 1.6, 2.4, 4)	5.39 (m)		5.51 (m)
	H-4	5.53 (br dd, <i>J</i> = 1.2, 4)	5.50 (m)		5.39 (m)
	H-5	4.80 (br ddd, <i>J</i> = 1.2, 8, 11)	4.81 (m)		4.79 (m)
	H-6	4.32 (dd, <i>J</i> = 8, 11)	4.31 (m)		4.40 (m)
	H-6'	4.93 (t, <i>J</i> = 11)	4.82 (m)		4.70 (m)
Ring B	H-1	5.18 (s)	5.15 (s)	4.72 (d)	4.89 (d, <i>J</i> = 1.4)
	H-3	6.53 (s)	6.48 (s)	6.26 (d)	6.21 (d, <i>J</i> =1.4)
Galloyl ³		7.03 (s)	7.15 (s)		7.14 (s)

HHDP ³		6.49 (s)		7.07 (s)		7.02 (s)
		6.58 (s)		6.63 (s)		6.62 (s)
Ring A	H-2			7.17 (s)		7.21 s)

1. Okuda et al. (1986).
2. Okuda et al. (1982).
3. Nawwar et al. (1994).

Table 4.9. ^{13}C -NMR data of compound **2** in acetone- d_6 plus D_2O (referenced relative to solvent peak = 29.92 ppm) with comparison to existing literature. (Appendix: NMR spectra of compound **2**).

Moiety	C	NMR shifts			
		1 a (Ref ¹)	Current work	1b (Ref ¹)	Current work
Glucose	C-1	90.81	91.07	91.76	91.90
	C-2	69.89	70.05	70.37	70.65
	C-3	63.27	63.36	62.28	62.40
	C-4	65.85	65.90	66.78	66.84
	C-5	72.55	72.61	73.14	73.22
	C-6	63.64	63.68	63.80	63.92
Ring B	C-1	46.05	45.92	51.89	51.84
	C-2	154.32	154.20	149.09	148.98
	C-3	128.59	128.74	125.01	125.34
	C-4	191.73	192.04	194.80	194.88
	C-5	96.14	96.25	92.37	92.42

	C-6	92.37	92.19	108.00	108.92
	C=O		165.86 ^a		165.72 ^a
Galloyl ³	1	118.7	119.82		118.88
	2,6	109.0	110.46		110.64
	3,5	145.6	145.98		140.61
	4	139.0	140.13		140.05
	C=O	164.9	165.55 ^a		165.36 ^a
HHDP ³	1, 1'	123.0, 123.9	124. 11 ^b , 125.21 ^b		124.26 ^b , 125.62 ^b
	2, 2'	106.1, 106.9	109.58, 107.66 ^c		110.19, 107.55 ^c
	3, 3', 5, 5'	143.9, 144.2, 144.8, 144.9	144.66 ^d , 145.45 ^d		144.95 ^d , 145.18 ^d
	4, 4'	135.4, 135.6	137.83, 136.35		137.59 ^e , 136.32
	6,6'	115.6, 115.8	117.39, 115.33		117.32, 115.24
	C=O	166.8, 167.3	166.39, 168.74		166.38, 168.77

Ring A	1		115.69		119.82
	2		119.82		116.24
	3		113.48		113.37
	4		145.79		147.72
	5		139.24		137.76 ^e
	6		143.27		147.04
	C=O		165.30 ^a		164.92 ^a

1. Okuda et al. (1986).
 2. Okuda et al. (1982).
 3. Nawwar et al. (1994).
- a, b, c, d, e: Shifts are interchangeable between each other.

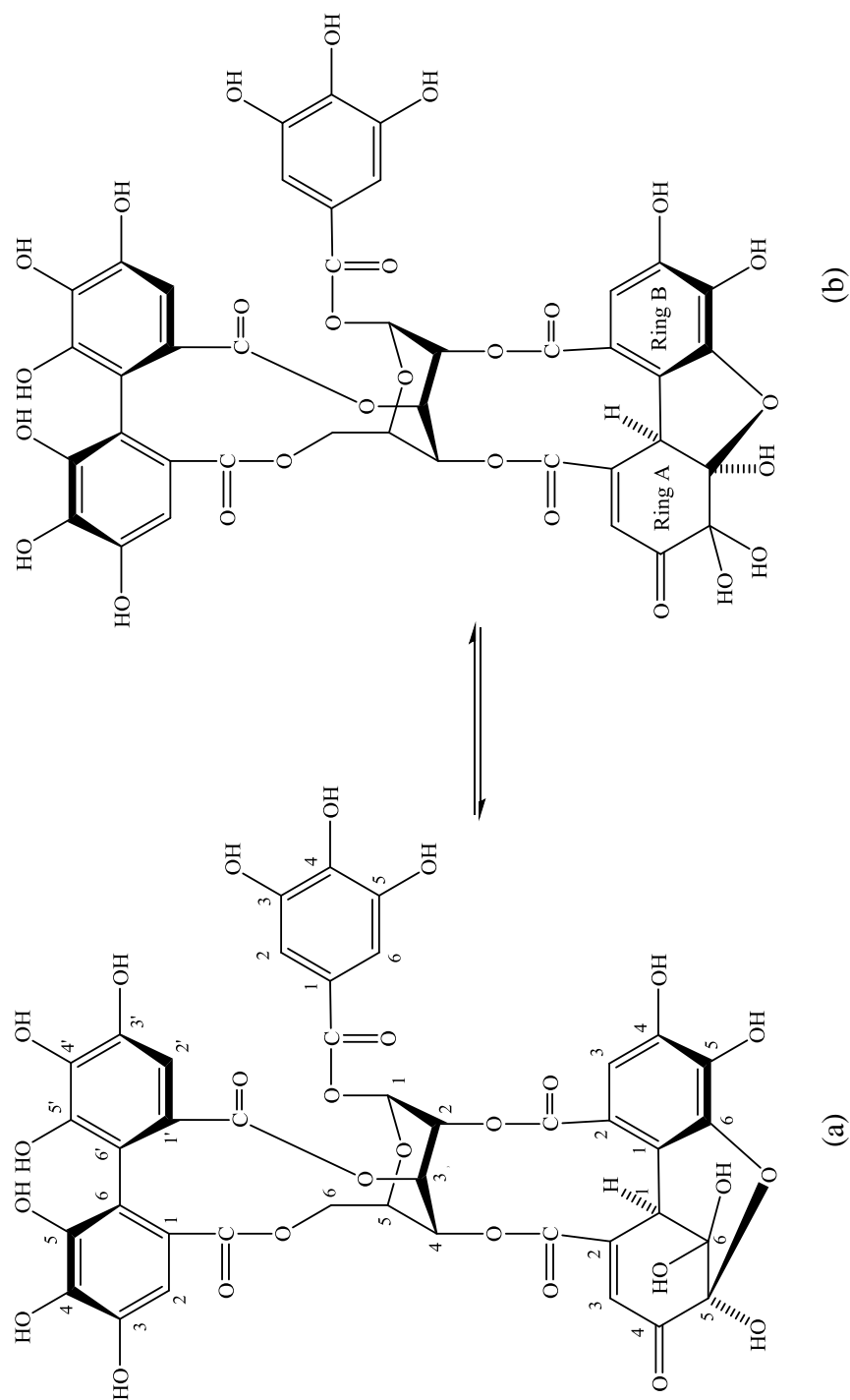


Figure 4.14. Major compound in F5: Compound **2** (Geraniin); equilibrium mixture of (a) and (b) upon mutarotation of the HHDP moiety in aqueous solution.

4.7 Cytotoxicity and Cytoprotection of F5 Against HepG2 Cell Lines

The potential cytoprotective effects of both the ethanol extract and F5 against cell death induced by *t*-BHP were evaluated in HepG2 cells. EC₅₀ values for cytoprotection of the ethanol extract, F5 and quercetin both for the direct activity and indirect activity are tabulated in Table 4.10. From the EC₅₀ values, it can be concluded that both ethanol extract and F5 are less active than quercetin in both of the analysis with F5 being 7-fold and 3-fold less active than quercetin in the direct and indirect assay, respectively. Viability of HepG2 cells exposed to 100 µg/mL of quercetin was reduced to 62.7 % in the direct assay (Figure 4.15c). Both the ethanol extract and F5 were non-toxic alone in all the concentration tested (6.25 – 100 µg/mL) and also protected the cells against cell death in a dose-dependent manner, with F5 being more potent than the ethanol extract (Figure 4.15a and 4.15b).

However, in the indirect cytoprotection assay, it was observed that quercetin was not toxic to the cells at the highest concentration (100 µg/mL) under the experimental protocol adopted (Figure 4.16c). Quercetin protected against *t*-BHP toxicity, with less potency than in the direct activity assay with cell viability of 98.0 % with treatment of 100 µg/mL. Both the ethanol extract and F5 were also non-toxic to cells alone. They also protected the cells against insults induced by *t*-BHP, with F5 being more potent than the ethanol extract.

Cell viability after being induced by *t*-BHP was observed at 43.6 % and 82.7 % with protection from 100 µg/mL of the ethanol extract and F5, respectively (Figure 4.16a and 4.16b).

Table 4.10. EC₅₀ values of cytoprotection activity of the ethanol extract, F5 and quercetin

Sample	Direct activity (µg/mL)	Indirect activity (µg/mL)
	Mean ± SEM	
Ethanol extract	89.1 ± 9.2	> 100
F5	46.5 ± 12.0	66.0 ± 7.6
Quercetin	6.6 ± 2.4	19.5 ± 0.2

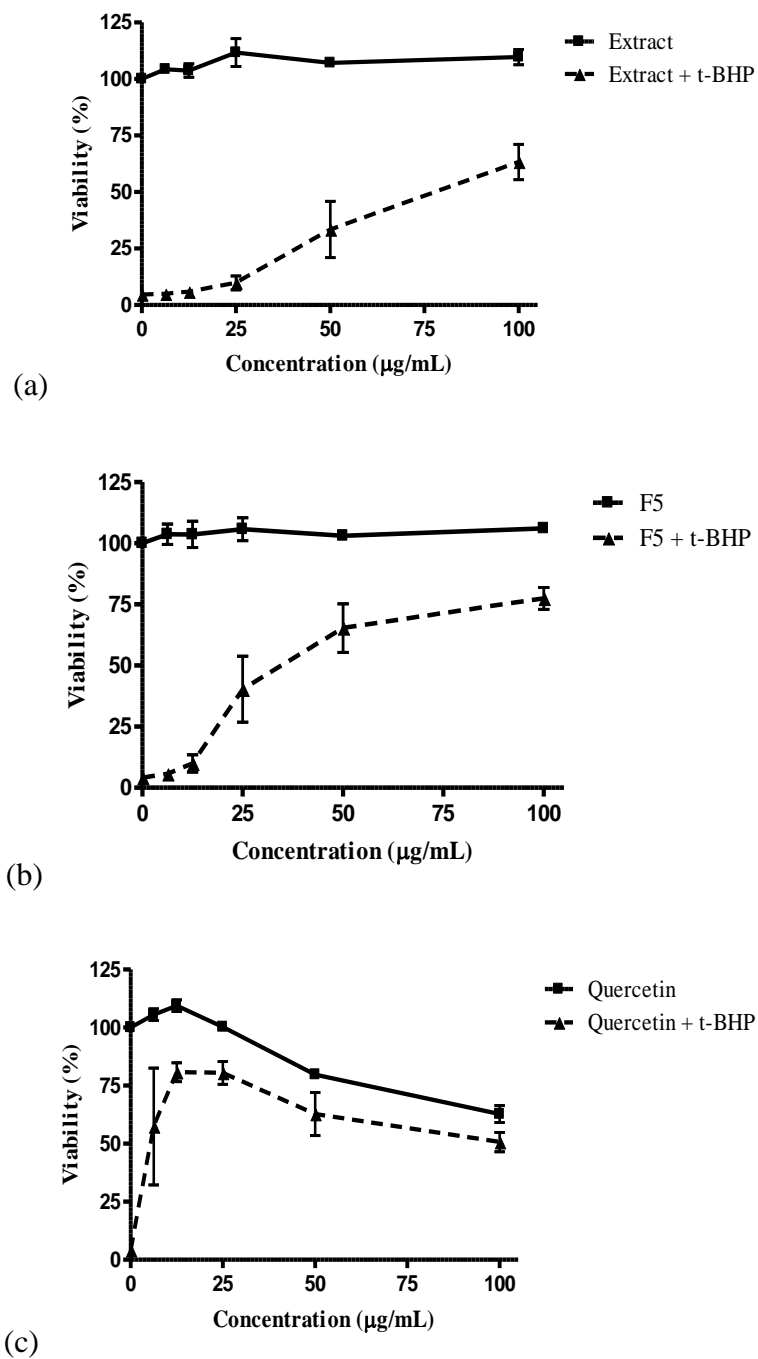


Figure 4.15. Direct cytoprotection activity of the (a) ethanol extract, (b) F5 and (c) quercetin on *t*-BHP induced HepG2 cells.

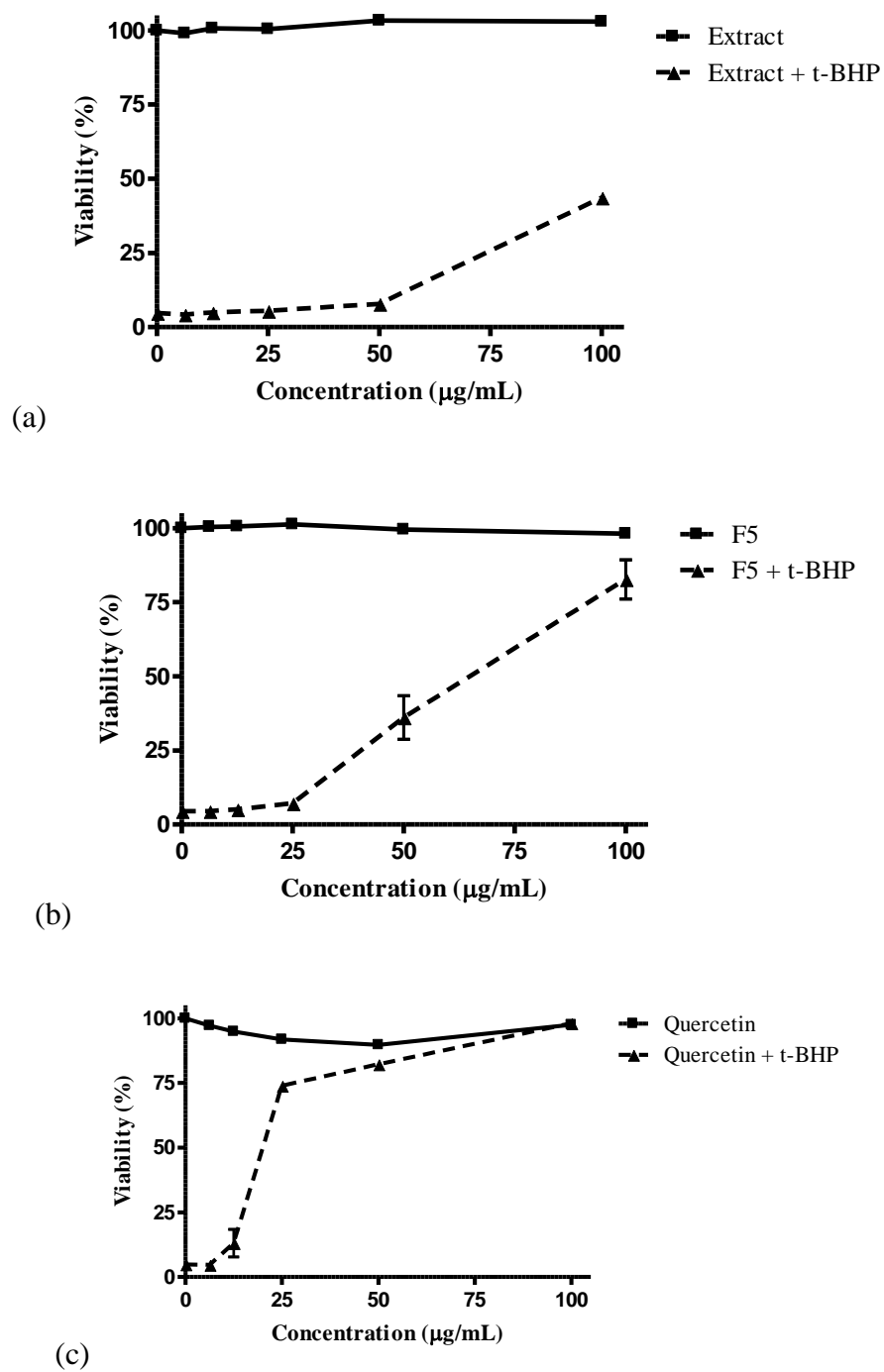


Figure 4.16. Indirect cytoprotection activity of the (a) ethanol extract, (b) F5 and (c) quercetin on *t*-BHP induced HepG2 cells.

4.8 Surface Morphology of *Staphylococcus aureus* by Field Emission Scanning Electron Microscopy (FESEM)

To illustrate surface morphology of the bacterial cell wall upon treatment with the samples, FESEM analysis were performed on *Staphylococcus aureus* (ATCC 11632) and MRSA. The control cells without any treatments were full and smooth on the surfaces, as cell walls are undisrupted and well formed (Figure 4.17). Treatments with the MIC dose of the ethanol extract (1500 µg/mL) disrupted the cell wall integrity of *Staphylococcus aureus* as surface deformation was observed (Figure 4.18). MRSA cells were observed adhering to each other with biofilm forming on the cells suggesting that some amounts of intracellular materials are released as observed in Li et al. (2011). Total collapse and broken cells were observed with the treatment with MIC dose of F5 on both *S. aureus* (ATCC 11632) and MRSA, as cell debris and indentation of the cell surface were seen (Figure 4.19). This suggests total lysis of the bacteria leading to bacterial death.

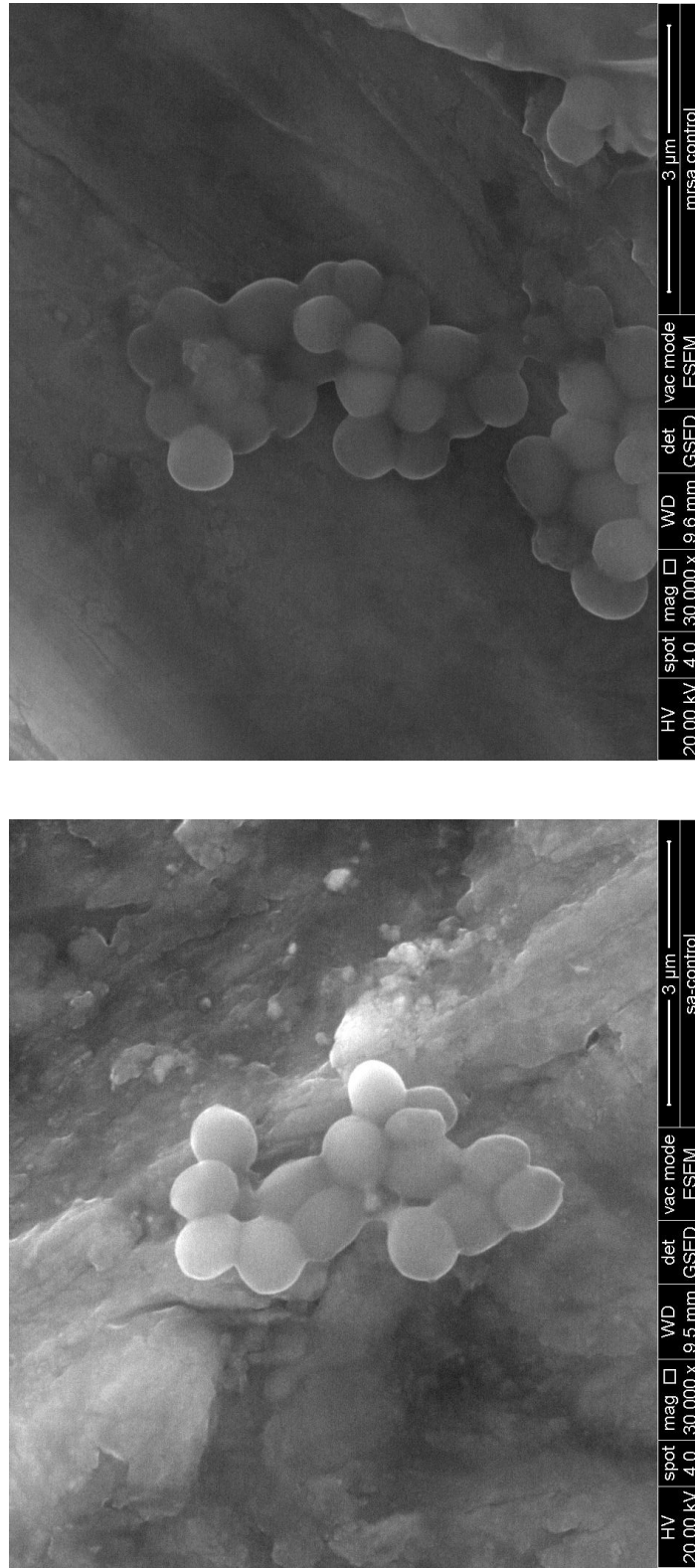


Figure 4.17. Morphology of the cells observed with FESEM of control (in MHB, without any treatment). Left:

Staphylococcus aureus, Right: Methicillin Resistant *Staphylococcus aureus*. (Magnification: 30000x)

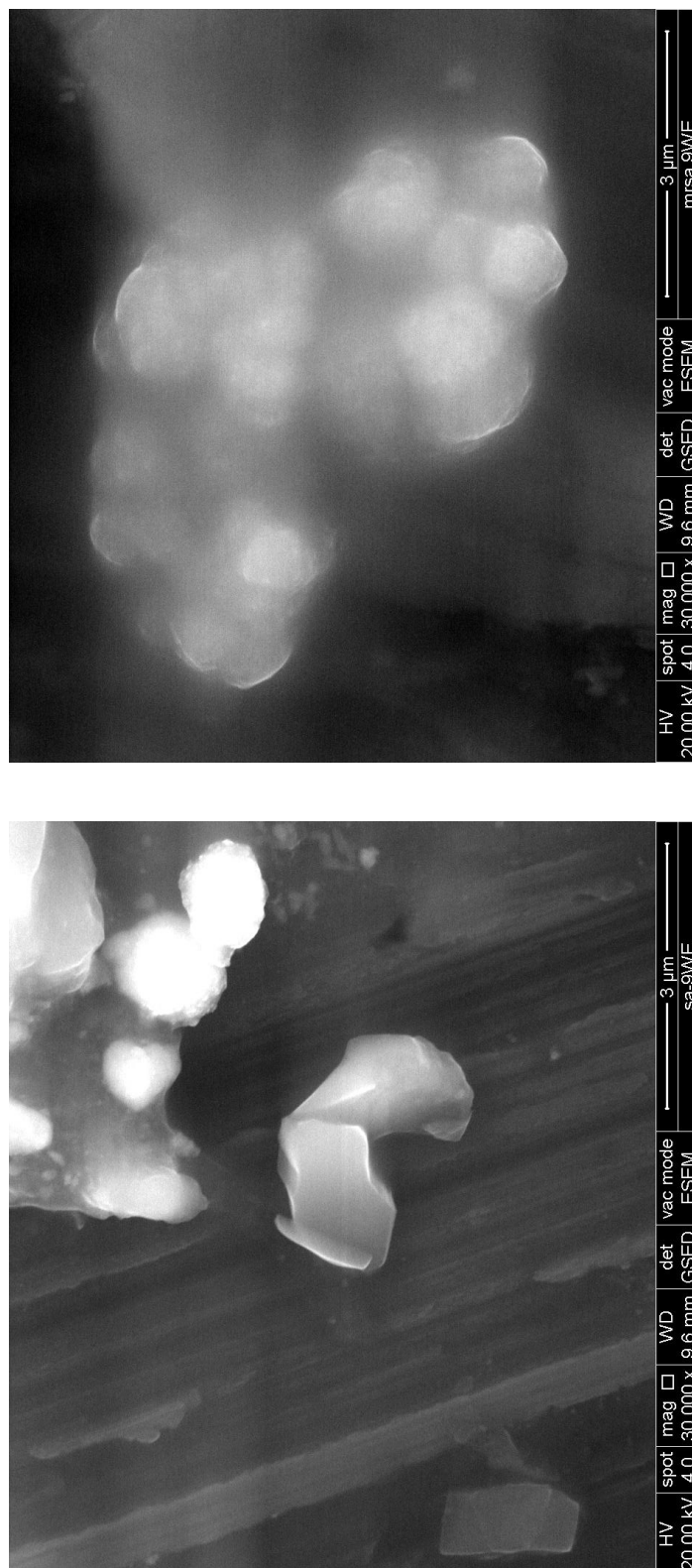


Figure 4.18. Morphology of the cells observed with FESEM after treatment with ethanol extract of *Acalypha wilkesiana* var. *macafeana* hort.. Left: *Staphylococcus aureus*, Right: Methicillin Resistant *Staphylococcus aureus*. (Magnification: 30000x)

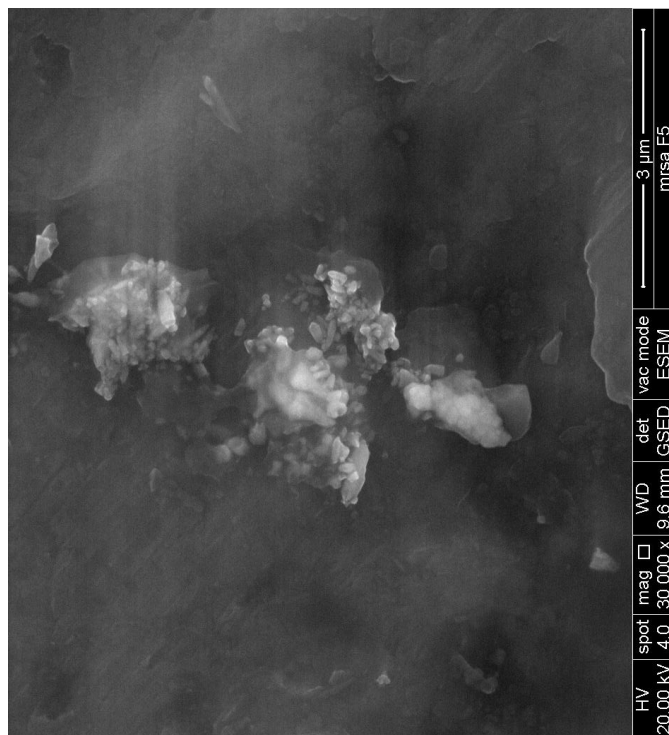
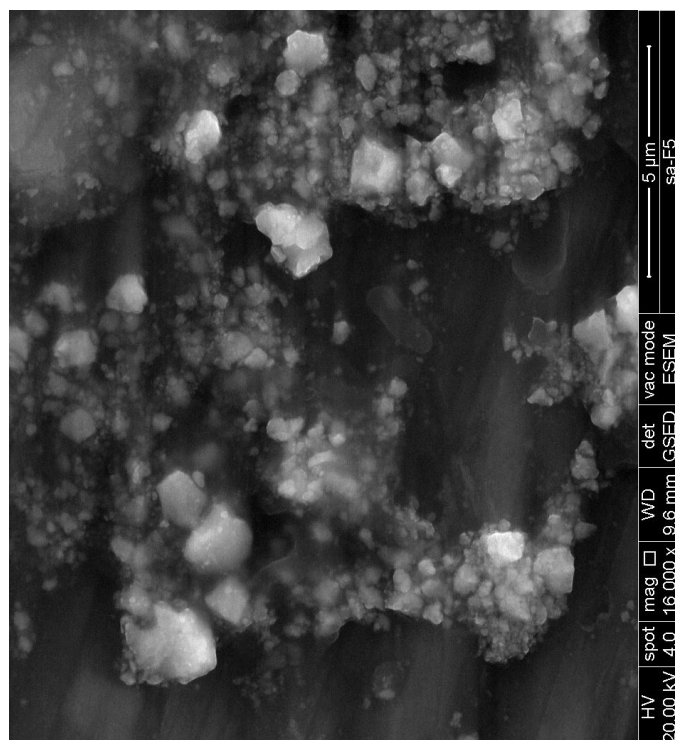


Figure 4.19. Morphology of the cells observed with FESEM after treatment with F5 from *Acalypha wilkesiana* var. *macafeana* hort. Left: *Staphylococcus aureus*, (Magnification: 16000x) Right: Methicillin Resistant *Staphylococcus aureus*. (Magnification: 30000x)

4.9 Synergistic Studies with Antibiotics against *Staphylococcus aureus*

To get a better understanding of the mechanism underlying the bactericidal activity of F5, we examined the effect of F5 with 3 antibiotics i.e., ampicillin, tetracycline and streptomycin via the checkerboard assay and time-kill assay. Results obtained indicated that the F5 was synergistic with ampicillin (Figure 4.20b) against Gram-positive *Staphylococcus aureus* (ATCC 11632) as represented by a convex line extrapolated from all the doses tested in the checkerboard assay. However, no synergy was observed between F5 and streptomycin (Figure 4.20a) and between F5 and tetracycline (Figure 4.20c) as additive relation was observed.

The bactericidal effect of F5 was further assessed in the time-kill assay whereby combining $\frac{1}{4}$ MIC of ampicillin (12.5 $\mu\text{g/mL}$) with $\frac{1}{2}$ MIC concentration of F5 (375 $\mu\text{g/mL}$) inhibited the growth of *Staphylococcus aureus* (Figure 4.21b). The synergy effect was further evidenced by the fact that $\frac{1}{4}$ of MIC concentration of ampicillin alone and $\frac{1}{2}$ MIC concentration of F5 alone were inactive. In brief, ampicillin and F5 work synergistically to inhibit *Staphylococcus aureus* (ATCC 11632). The combination of F5 and streptomycin (Figure 4.21a) was not boosting the bactericidal potencies of F5. Likewise with tetracycline (Figure 4.21c), as the combination, F5 alone and

tetracycline alone produces growth curves that are very similar. In conclusion, F5 is specifically synergistic with ampicillin.

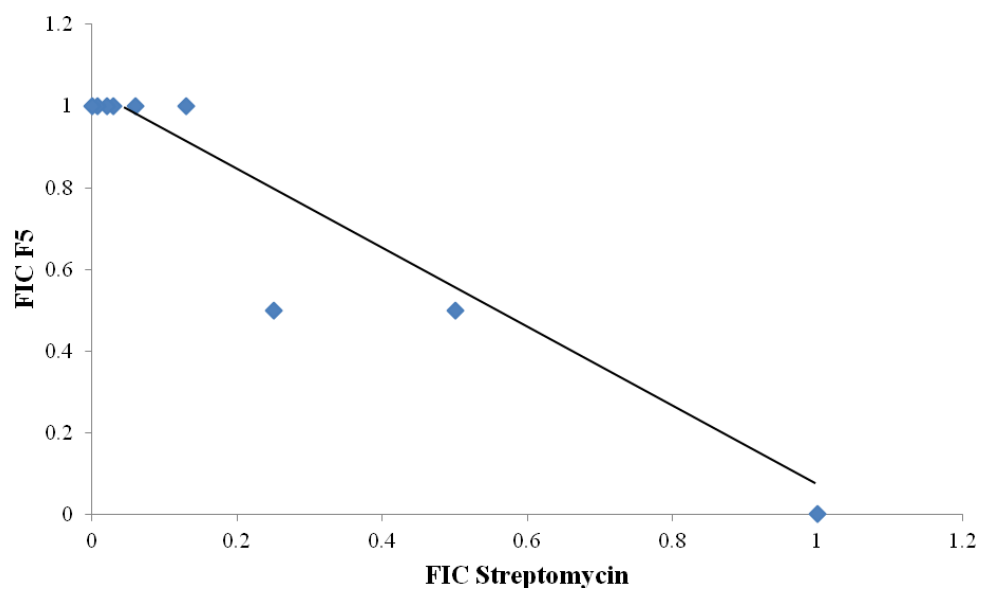


Figure 4.20 (a). Isobologram of Streptomycin in combination with F5 which shows additive interaction.

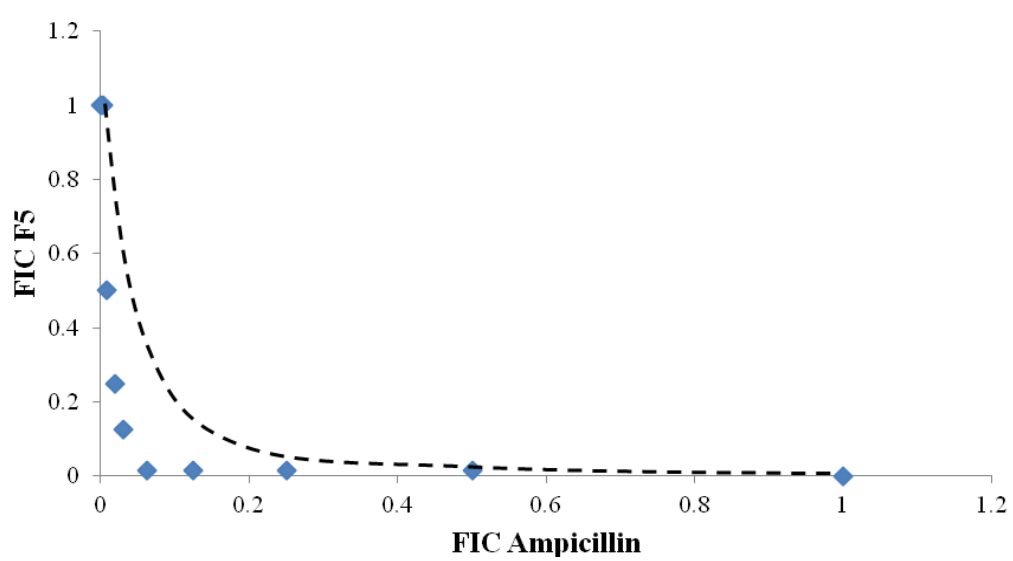


Figure 4.20 (b). Isobologram of Ampicillin in combination with F5 which shows synergy interaction.

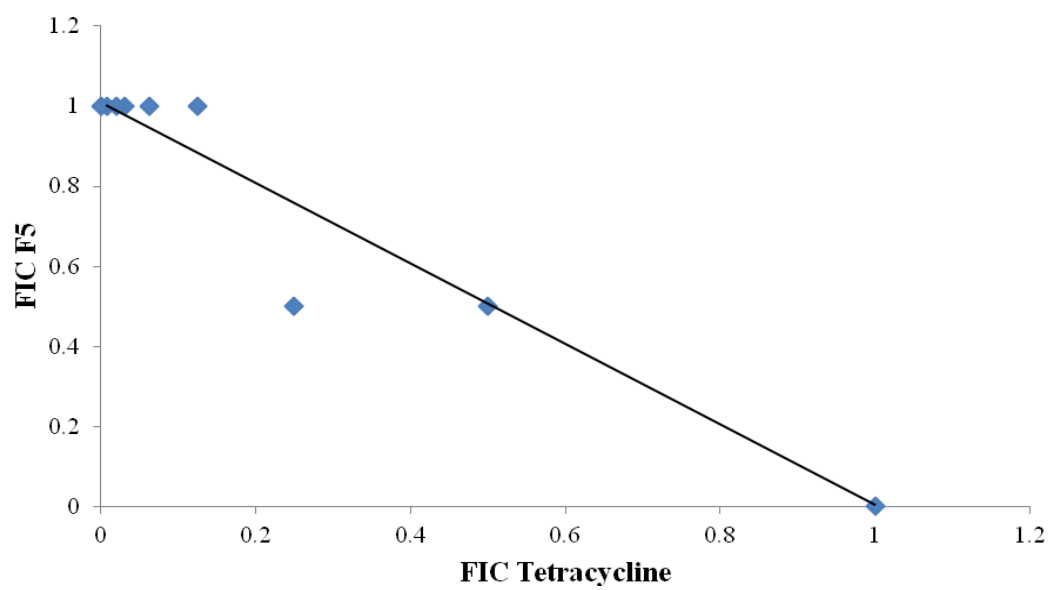


Figure 4.20 (c). Isobologram of Tetracycline in combination with F5 which shows additive interaction.

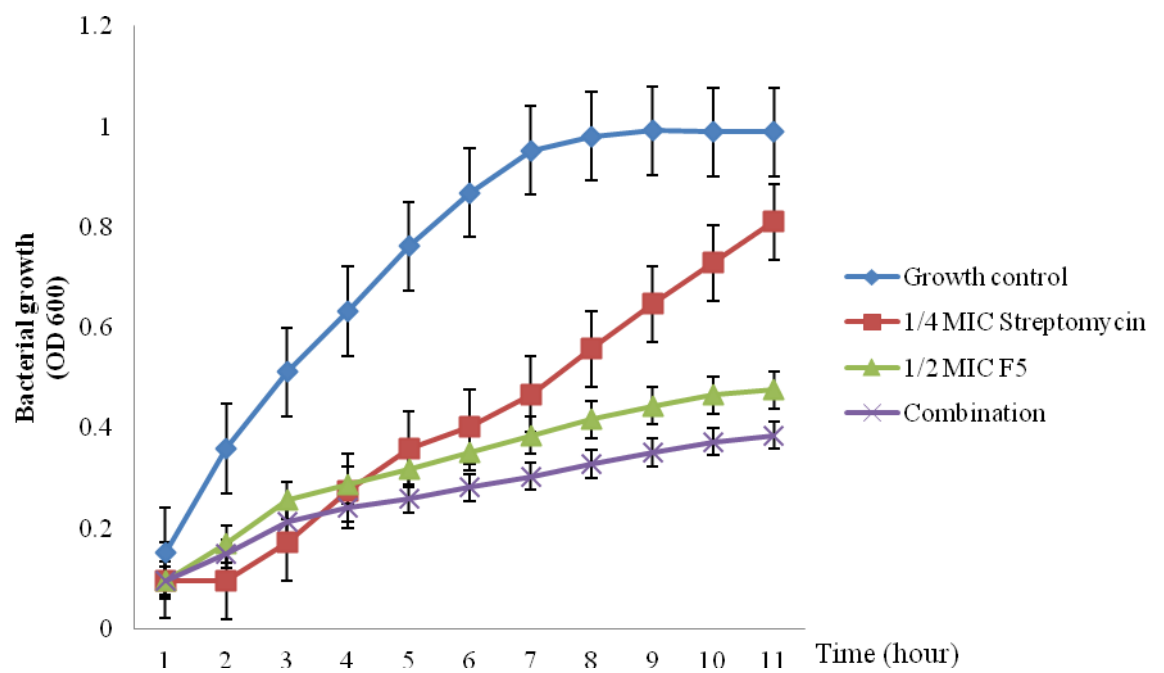


Figure 4.21 (a). Graph showing time-kill curves of combination of Streptomycin with F5 against *Staphylococcus aureus* (ATCC 11632).

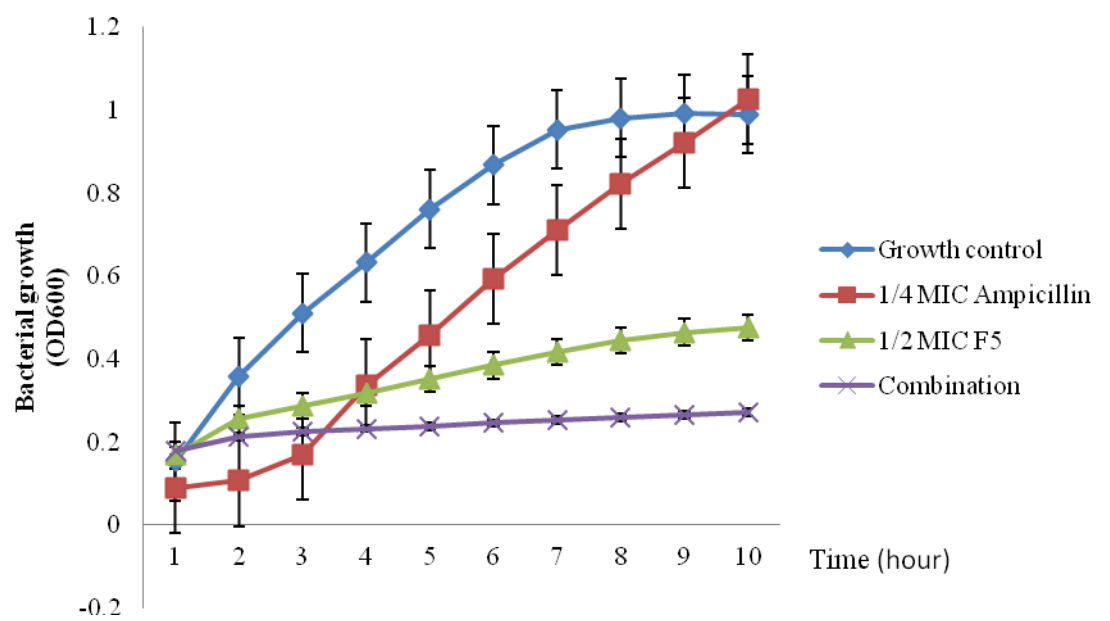


Figure 4.21 (b). Graph showing time-kill curves of combination of Ampicillin with F5 against *Staphylococcus aureus* (ATCC 11632).

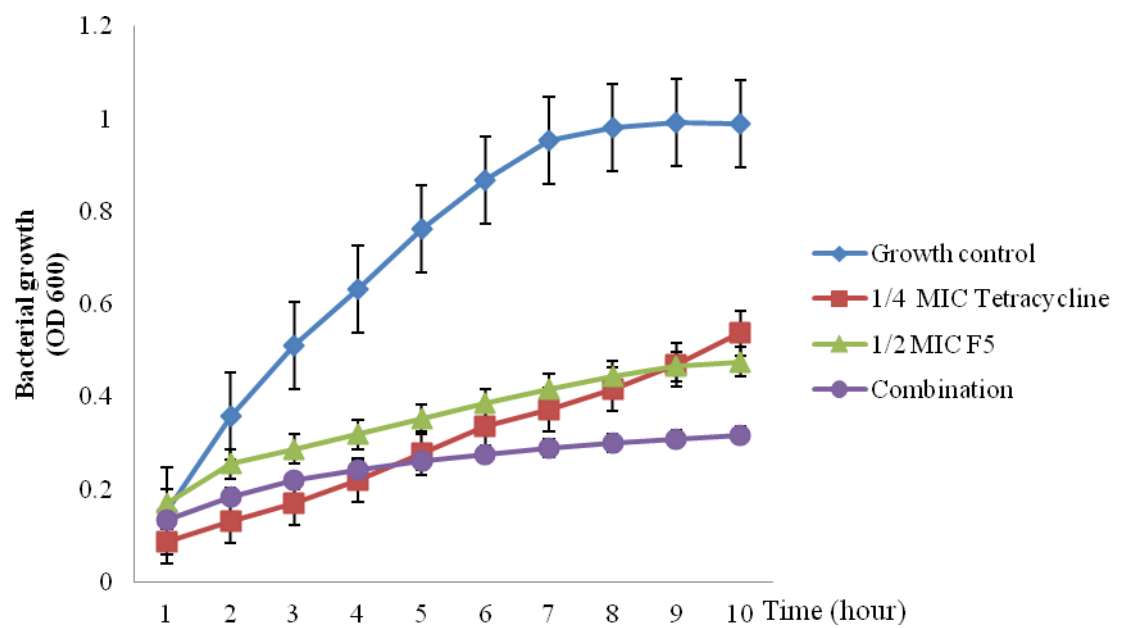


Figure. 4.21 (c). Graph showing time-kill curves of combination of Tetracycline with F5 against *Staphylococcus aureus* (ATCC 11632).

4.10 Formulation of F5 as a Topical Antibacterial Formulation

Three formulations were assessed for the antibacterial properties by the pour plate well diffusion test against *Staphylococcus aureus* (ATCC 11632). The pour plate well diffusion test provides a reliable and standardized method to assess the antibacterial activity of creams, ointments and formulations of anti-infectives against bacteria strains (Rodeheaver et al., 1980; Conly et al., 1985; Ordoñez et al., 2009). As shown in Table 4.11, noticeable antibacterial activities were observed with formulation 1 with inhibition zone diameters of 9.01 ± 0.14 mm. No inhibition was observed with the other two formulations. Formulation 1 consisted of 60 % water with a white soft paraffin and liquid paraffin base.

The efficacy of the active tannin compounds in the formulation was observed as the test was repeated until day 10 after the formulation was prepared. As for the 10 days, the same antibacterial potencies were observed from formulation 1. Indeed, the inhibition diameters observed were 10.05 ± 0.46 mm, 9.39 ± 0.22 mm, and 9.17 ± 0.96 mm, respectively for day 3, day 7 and day 10. This was likewise observed with the anti-infective Betadine[®] and Burnol[®] which were used as positive control.

Table 4.11. *In-vitro* plate inhibition zones of formulation

Sample	Inhibition zones (mm)			
	Day 1	Day 3	Day 7	Day 10
Formulation 1	9.01 ± 0.14	10.05 ± 0.46	9.39 ± 0.22	9.17 ± 0.96
Placebo 1	NI	NI	NI	NI
Formulation 2	NI	NI	NI	NI
Placebo 2	NI	NI	NI	NI
Formulation 3	NI	NI	NI	NI
Placebo 3	NI	NI	NI	NI
Betadine [®]	13.01 ± 0.42	14.06 ± 0.36	12.81 ± 0.37	14.35 ± 0.56
Burnol [®]	12.18 ± 0.46	11.86 ± 0.34	11.87 ± 0.34	11.42 ± 0.33

* NI: No inhibition observed.

4.11 *In vivo* Assessment of the Formulation against Skin Infection of *Staphylococcus aureus* on Guinea Pigs

To further assess the effectiveness of the formulation against *Staphylococcus aureus* infections, the formulation (dose) were tested *in vivo* on guinea pigs that were infected with the afore mentioned bacterium. The viable colony counts 10 days post infection are tabulated in Table 4.12 and the wound healing properties are captured in Figures 4.22 (a-c). Viable colony counts after treatments shows that colonies from wounds with treatment of formulation 1 is less than wounds without treatment and treatment with placebo, but wounds with treatment with Burnol[®] was with the least viable colony. Although the viable counts show a reduction with treatment of formulation 1, the wound that was infected with the bacterium and did not receive any treatment, did not show any symptoms that were common with *Staphylococcus aureus* infections such as formation of blisters containing abscess, red blistering breaks and formation of crusts.

As for the wound healing ability of the formulation, it is observed as illustrated in Figure 4.22 (a-c), that the wound with treatment with formulation 1 healed faster than wounds that were treated with both Burnol[®] and placebo. Formulation 1 promoted effective wound healing property than Burnol[®]

suggesting that the tannins (the active ingredient) plays a role in this instance. This would suggest that the antioxidant property of the formulation plays a role in the wound healing ability of the formulation.

Table 4.12 *Staphylococcus aureus* count after treatment

Treatments	<i>Staphylococcus aureus</i>
	(cfu/mL)
Formulation 1 (with tannin fraction)	9.5×10^3
Formulation 1 placebo	4.4×10^4
Burnol [®]	3.4×10^3
Without treatment	2.1×10^4

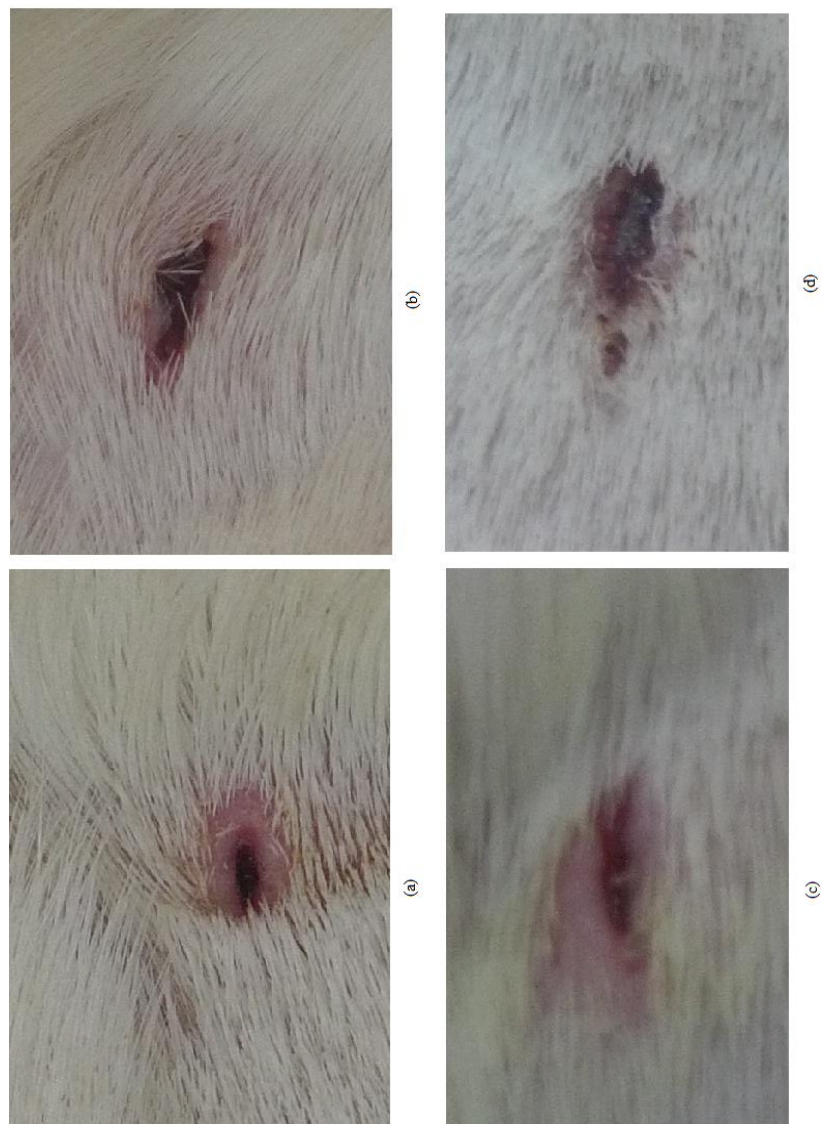


Figure 4.22 (a). Treatment results at Day 4 after inoculation of bacteria. (a) Formulation 1, (b) Placebo, (c) Burnol[®] and (d) No treatment.

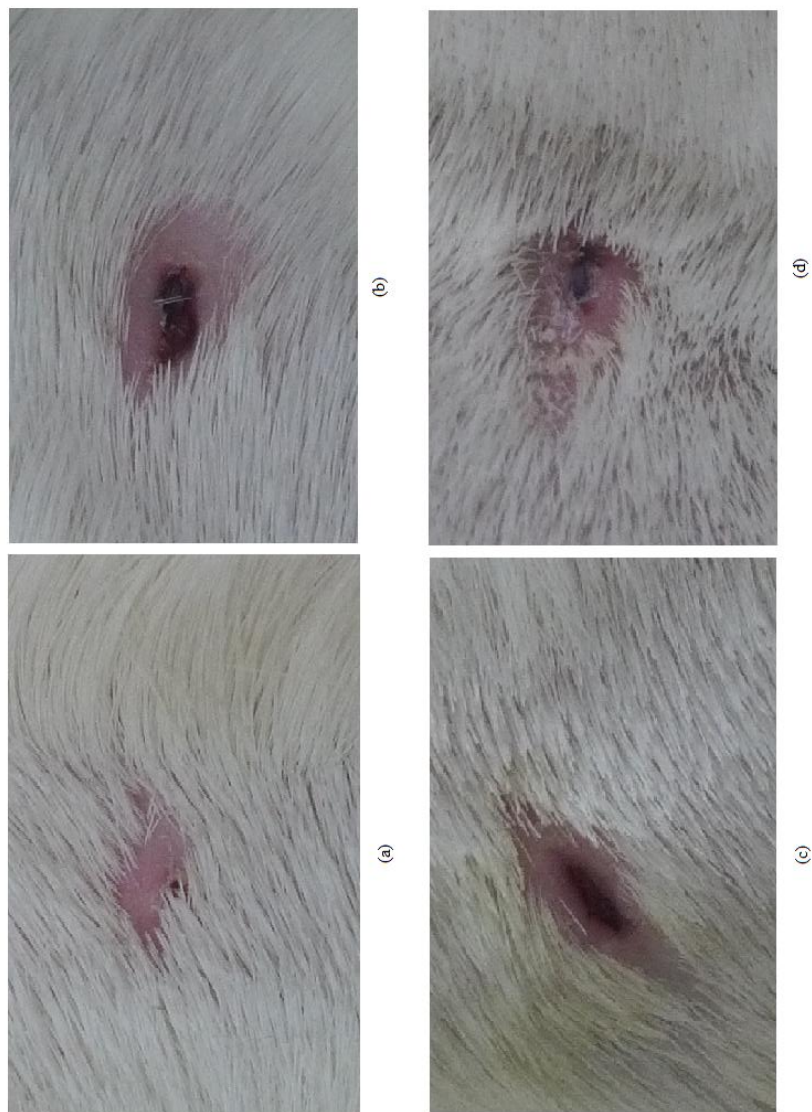


Figure 4.22 (b). Treatment results at Day 6 after inoculation of bacteria. (a) Formulation 1, (b) Placebo, (c) Burnol[®] and (d) No treatment.



Figure 4.22 (c). Treatment results at Day 10 after inoculation of bacteria. (a) Formulation 1, (b) Placebo, (c) Burnol[®] and (d) No treatment.

CHAPTER 5

DISCUSSION

The significance and importance of natural product as a source of drug discovery is highlighted with the increasing rate of introduction of new chemical compounds as possible drug candidates and as templates for synthetic compounds, the number of diseases that is treated and prevented by natural products, as well as the frequency of it being use in treatment (Rout et al., 2009). The approach of selecting plants for investigation for drug discovery through ethnopharmacological claims remain one of the most frequently used approach than random selection (Rout et al., 2009). In this research, we have selected our plant with the foundation of its ethnopharmacological claims as well as previous research findings, and proceed with more depth and strategy on gaining increased knowledge in natural product as a source of drug discovery.

5.1 *Acalypha wilkesiana* var. *macafeana* hort. (Euphorbiaceae Juss.)

Acalypha wilkesiana var. *macafeana* hort. (Euphorbiaceae Juss.) was selected for study due to its abundance in distribution in Malaysia and other tropical countries and the fact that it is seasonal all year round. This plant is also used

as an ornament. Despite the ethnopharmacological claim that sets the foundation of selection for this plant, it also has published results which complement its traditional uses. We set upon the research to investigate in more depth on the potential possibility of this plant as an antioxidant and antimicrobial agent.

Several lines of evidence point to the fact that members of the genus *Acalypha* L. elicit several biological properties such as antioxidant (Badami & Channabasavaraj, 2007; Iniaghe et al., 2008), antimicrobial (Jebakumar et al., 2005; Mothana et al., 2008), and anticancer (Madlener et al., 2010; Shin et al., 2012). Several species in this genus have been tested clinically (Oyelami et al., 2003). In all of the research aforementioned, phytochemical studies show that these properties are due to the secondary metabolites that are in abundant in this species such as terpenes, flavonoids and tannins (Amakura et al., 1999; Adesina et al., 2000). Understanding the background of a certain plant genus and species provides some lead to natural product study. Literature review is essential in setting the template of research although some scientists prefer random selection of plants.

5.2 Extraction of the Bioactive Secondary Metabolites from the Plant

Extraction of natural product is crucial, considering that this is the first step in gaining the bioactive constituents from the plant of interest. Choosing a solvent for extraction is important as the solvent has to be able to extract constituents in interest of the study, or extract out as much compounds as possible, depending on the objective of the research (Ghisalberti 2008). The more efficient the extraction step, the greater the range of compounds present in the extract (Ghisalberti 2008). In this research we extracted air-dried material of the plant with sequential maceration from low to high polarity of solvent. Sequential extraction has been proved to give higher yield than using single solvents (Bazykina et al., 2002; Walia et al., 2011). This also provides a rough layout for screening of the crude extract for biological properties as it separates low polarity to higher polarity compounds. For an example, ethanol extracts out polar, water-soluble compounds as hexane extracts out non-polar compounds and fatty acids (Bazykina et al., 2002; Ghisalberti 2008). Extraction with water might extract out saponins and glycosides (Kalaivani & Mathew 2009).

5.3 Biological Evaluations of the Crude Extract and Fractionation

Screening of the antioxidant and antimicrobial properties of the crude extracts of *A. wilkesiana* var. *macafeana* hort. both evidenced that the ethanol extract gives the highest results for both biological activities. The hexane fraction did not exhibit any antibacterial properties against any bacterial strains tested. Hence, fractionation was executed on the ethanol extract to further locate the bioactive compound. Extraction with ethanol usually pulls out highly polar, water-soluble compounds such as aromatic phenolics, flavonoids, catechols, glycosides and tannins (Bazykina et al., 2002).

Fractionation was commenced by an open column chromatography with Sephadex LH-20. Sephadex LH-20 is a crosslinked dextran gel that has both lipophilic and hydrophilic properties owing to the isopropyl groups of the hydroxypropylation and hydroxyl functions present (Henke 1995). The exclusion limit of Sephadex LH-20 at maximum swelling i.e., in dimethylsulfoxide (DMSO) is 4000, which means, all compounds with a molecular mass of more than 4000 elutes without separation as it only passes through the interparticle spaces (Henke 1995). Other compounds elutes in decreasing molecular size. Besides size exclusion, Sephadex LH-20 can also be used for adsorption chromatography (Henke 1995).

Our fractionation method used water/ethanol and water/acetone composition to take advantage of both exclusion characteristics of the gel and also the adsorptive separation (Henke 1995). Fraction 1 to fraction 4 were eluted out with water/ethanol mixtures in account to size exclusion, whereby the last fraction (F5) was eluted out with water/acetone by adsorptive separation as it was retained in the gel with water/ethanol mixture. Sephadex LH-20 sorbs tannins in alcohol and releases them with acetone. Hence, water/acetone mixtures were used to elute out tannins that are retained by Sephadex LH-20 (Hagerman 2002). According to the Tannin Handbook of Hagerman (2002), Sephadex LH-20 is very useful in separating tannin to non-tannin phenolics.

5.4 Antioxidant Properties of Fractions from the Ethanol Extract

As part of an ongoing study in the Natural Product Team of our faculty to identify antioxidants from Asian medicinal plants (Wijaya et al., 2011a; Wijaya et al., 2011b), we have previously isolated flavonoid glucosides, flavanones and pyrrolizidine alkaloids. One of the largest groups of flowering plants is the family Euphorbiaceae Juss. which encompasses about 300 genera and 7500 species (Wiart 2006) of which many are considered as medicinal plants. In this context, it is worth to acknowledge that illnesses linked to oxidative stress are traditionally treated by members of the genus *Acalypha* L. such as *Acalypha racemosa* Wall. ex Baill., *Acalypha indica* Linn. and

Acalypha ornata Houchst. Ex A. Rich. thus these species have attracted the attention of researchers (Iniaghe et al., 2008; Kavitha et al., 2009; Joy et al., 2010; Onocha et al., 2011; Mathew et al., 2011). The notion of *Acalypha* L. alleviating illnesses induced by oxidative stress is further supported by an analysis of an unidentified species used to treat breast cancer and inflammation (Bussing et al., 1999; Lim et al., 2011). However, little attention has been focused on the antioxidant properties of members of the vast genus *Acalypha* L.

With this premise in mind we have examined the antioxidant potencies of the Malaysian medicinal shrub *Acalypha wilkesiana* var. *macafeana* hort used to heal wounds, skin disorders and infections. To examine the antioxidant effects of *Acalypha wilkesiana* var. *macafeana* hort., we tested an ethanol extract using a panel of conventional tests which evidenced significant antioxidant and cytoprotective capacities. In order to identify the antioxidant principle, five fractions (F1-F5) from the ethanol extract were tested and F5 displayed robust antioxidant and cytoprotective effects. In natural product research, it is a practice to compare results of the active fraction(s) with the crude extract which it is was extracted out. This practice is important to observe whether the active fraction is really more potent than the crude extract or otherwise. In some instances, a single compound isolated out gives a less potent activity due

to synergy of more than one compound in the extract. Thus, comparison on crude with the isolated active fraction or compound is crucial to make sure the research is on track and not misleading.

Several *in vitro* antioxidant techniques have been developed such as FRAP, DPPH and BCB assays. Indeed, one ought to perform several methods to accurately estimate the antioxidant potential of a sample as it should cover the mechanisms of different reactions (Huang et al., 2005). Recently, Moon and Shibamoto (2009) suggested combining assays associated with lipid peroxidations and assays associated with radical and electron scavenging in order to evaluate antioxidant capacity. In line with this contention, we analyzed our samples with three different assays to accommodate different antioxidant mechanisms.

In order to evaluate the antioxidant potential of the ethanol extract of *Acalypha wilkesiana* var. *macafeana* hort. and its fractions, three antioxidant tests were performed; the FRAP assay, the DPPH radical scavenging assay and the β -carotene bleaching assay. The total phenolic content of the samples was also assessed using the Folin-Ciocalteu reagent. Interestingly, F5 displayed the most prominent antioxidant effect in all three assays as compared to other

fractions and the crude ethanol extract. The total phenolic content for F1 and F5 were significantly higher than the ethanol extract. Clear correlation is seen between the antioxidant potentials and the total phenolic content, thus suggesting that the phenolics account for antioxidant activity of the plant.

Several studies have shown that the mechanism underlying polar antioxidant involves reactions with the hydroxyl (OH) group present in phenolics (Duthie & Crozier 2000). Indeed, phenolics are composed of one or more aromatic rings bearing one or more hydroxyl groups and are therefore potentially able to quench free radicals by forming stabilized phenoxyl radicals (Tang et al., 2004) and most of the current antioxidants isolated so far from flowering plants are simple phenolic compounds which owe their properties to the mere fact that their aromatic hydroxyl moieties react with free radicals (Tang et al., 2004).

Given the involvement of hydroxyl (OH) groups, it is tempting to speculate that the total content of phenolic compounds could be seen as a standard step in the evaluation of antioxidant activities of plant extract. Indeed antioxidant capacities correlated with total phenolic content and DPPH reducing ability, in a study of traditional Chinese medicines (Dudonne et al., 2009). A comparative

antioxidant study of 30 plant extracts used in the nutraceutical industry highlighted a relationship between phenolics concentration in the plant extracts and their free radical scavenging and ferric reducing capacities (Hagerman et al., 1998).

5.5 Antimicrobial Properties of Fractions from the Ethanol Extract

According to Asian Network for Surveillance of Resistant Pathogens (ANSORP), MRSA infections accounts for 25.5 % of community-associated infections and 67.4 % of healthcare-associated infections in Asian countries (Song et al., 2011). In Malaysia, the annual MRSA infection rates ranged from 5.0 to 19.5 admissions for every 1000 admissions (Al-Talib et al., 2010). Therefore, the increasing ability of pathogenic bacteria to develop resistance to antibiotics, justify the search for “co-antibiotics” which could be very well originated from natural products of plant origin.

So the question arises on the significance of investing time and funds into characterizing antimicrobial substances from plants. Though the strengths of phytochemicals are less obvious, but it is certainly compelling due to four main reasons highlighted by Gibbons (2008). Firstly, their existence in plants for the very reason as a defense mechanism against soil and environment pathogens,

may very well be used against many Gram-positives and Gram-negatives bacterium. Secondly, in terms of phytotherapy, there are countless examples of plants being used either systemically or topically to treat bacterial infections. There have to be opportunities to investigate antibacterial of plant origin. Thirdly, nature's extensive pool of bioactives with ultimate chemical diversity which includes chiral structures and diverse functional groups engineered by the plants and last, the uniqueness of the phytochemical structure which are structurally different from microbial derived antibiotics might give a distinct mode of action from these existing compounds. All these arguments make strong points for researchers to continue to explore the field.

Pour plate disc diffusion assay is one of the methods to quantify the antimicrobial activity of a substance based on the size of inhibition zone. The pour plate disc diffusion method is appropriate for screening of antimicrobial activity due to the possibility of testing few samples against a single microorganism, and the advantage of small quantity of sample needed (Rios et al., 1988). According to Gislene et al., (2000), any chemical that demonstrates zones of inhibition of more than 7 mm are acceptable as an active antimicrobial agent. In our research, most of the crude and fractions showed inhibition zones of more than 7 mm against Gram-positive bacteria tested, with F5 inhibiting more than 8 mm zone on all the strains tested. Crude extracts exhibits lower inhibition zone. This could be explained by slow diffusion of

large molecules into the agar and insufficient active compounds present in the crude extracts (Taylor et al., 2001).

Though the pour plate disc diffusion method is a reliable method for screening preliminary antimicrobial compounds, it should never be used as a definitive method. Results of inhibition zones ought to be quantified with dilution assays (Rios et al., 1988). Dilution method involves homogenous dispersion of the sample in a microorganism selective culture media. Dilution method is necessary to determine the minimum inhibitory concentration (MIC) of compounds. Dilution assays are more laborious and requires more time, but it is often more precise. The dilution method is also the only method to identify the minimum bactericidal concentration (MBC) of a certain compound (Rios et al., 1988). To avoid sedimentation of the plant extract during incubation, shaking throughout the experiment is required.

Strong inhibitors have an MIC value lower than 500 $\mu\text{g/mL}$ and moderate inhibitors are inhibitors with MIC value between 600 and 1500 $\mu\text{g/mL}$. Inhibitors with MIC value above 1600 $\mu\text{g/mL}$ are recognized as weak inhibitors (Duarte et al., 2007; Wang et al., 2008). Pharmaceutical companies set a level of any phytochemicals with MIC's of greater than 100 $\mu\text{g/mL}$ as poorly active and are interested in pure phytochemicals with MIC's of ideally between 2 to 10 $\mu\text{g/mL}$ (Gibbons 2008). In our study, we observed that the

fraction F5 inhibits *Staphylococcus aureus* and MRSA at 750 µg/mL, thus categorizes F5 as a moderate inhibitor. F5 kills both *Staphylococcus aureus* and MRSA at 3000 µg/mL. This is interesting as F5 still consists of more than one compound and is not a pure substance. It is compelling to suggest that F5 might very well contain constituents which can exert strong inhibition against bacteria.

This study highlights that neither the crude nor the fractions are good inhibitors of Gram-negative bacteria. This was to be explained by the structure of Gram-negative bacteria. Gram-negative bacteria offer a more complex barrier system against permeation of foreign substances, in this case, the antimicrobial agents. This is attributed to the specialized cell wall structure and especially the presence of an outer envelope, which results impermeability of the bacterium against antibiotics (Denyer & Maillard 2002).

5.6 Identification of Constituents of F5

To gain further insights to the nature of active constituents of F5, high-performance liquid chromatography (HPLC) and liquid chromatography – mass spectrometry (LC-MS) analysis were performed allowing the identification of tannins. HPLC is an invaluable tool for analyzing natural products. Recent development of HPLC with small size column packings has

led to separation of compounds of closely related structures. Characterization and identification of tannins has been challenging due to the complexity, diversity and the large size of compounds. Multiple isomers of tannins can be present in one extract, thus creating more challenge in isolation of tannins.

In our study the most challenging obstacle was to obtain a good separation of all the compounds in F5 due to the complexity and diversity of tannins. Some hydrolysable tannins are observed to form multiple peaks in reversed phase HPLC analysis, thus making it more challenging to separate the compounds (Hatano et al., 1988). The multiple peaks are due to the molecule of dehydrohexahydroxy-diphenoyl (DHHDP) that are present in ellagitannins which forms an equilibrium mixture of isomeric hydrated hemiacetals (Hatano et al., 1988). This mixture exhibits several peaks due to components which are isomeric and are retained differently in the column (Hatano et al., 1988).

We also faced a challenge with optimizing a suitable solvent for HPLC analysis of the tannin fraction. Our observation is congruent with the findings of Hatano and colleagues (1988), as tannins which are dissolved in alcohol prior HPLC analysis, have remarkable changes in terms of peak shape and intensity in a time-dependant manner. We dissolved the sample in acetonitrile and prepared fresh samples to be used in the analysis for obtaining a standard chromatography profile of the tannin fraction. The wavelengths that are used

for detection of the compounds in the sample also have to be optimized in order to detect all of the compounds contained in the sample. As different wavelengths, detects different chromophores of a compound. In this research we opt to use 254 nm and 280 nm as it detected most peaks from the fraction. Both of these wavelengths detect chromophores of phenolics and tannins. Although these wavelengths detected the most compounds present in the fraction, there might be other compounds that are not visible with a UV/Vis detector, which are compounds that illuminates and are unable to be detected.

With current chromatography technology, separations of tannins are achievable, though isolation and identification of the compound is still a challenge. The requirement of an amount of sample for identification via Nuclear Magnetic Resonance (NMR) is sometimes unattainable due to the large molecular mass of tannins. Hence, a large mass of sample is needed in a purity that is acceptable for analysis of NMR. Liquid Chromatography - Mass Spectrometry (LC-MS) then play a vital role in identification of tannins. The main advantage of this technology is its requirement of a very small amount of sample, and the possibility of identifying the compounds without isolation.

LC-MS is recognized as a breakthrough in analysis and characterization of natural products with the synergy of both LC and MS. LC presents MS with cleaner samples, whereas MS helps reduce laborious sample preparation that

are usually required for analyte identification (Cheng et al., 2008). The combination of both of these powerful technologies makes it capable for accomplishing delicate and difficult separation and can be used to detect analytes with selectivity and sensitivity.

In this study, we used soft ionization method, electrospray ionization (ESI) in negative mode and Time-of-Flight (TOF) analyzer. ESI is atmospheric ionization process which causes deprotonation or protonation of analyte molecules to generate even-electron charged species which are much stable against further fragmentation. Ionization in the negative mode produce deprotonated molecule $[M-H]^-$, $[M-H]^{2-}$ or $[2M-H]^-$ ions (Salminen et al., 1999). TOF is the current analyzer which accelerates ions generated using a high voltage along a vacuumed tube. In the accelerator, small ions would move faster compared to larger mass, thus arrives at different times depending on their m/z . The TOF analyzer has no mass limit range, thus it is suitable for analyzing high molecular weight compounds such as tannins.

The major tannin was identified as geraniin. Geraniin is the main tannin of plants of the Euphorbiaceae family (Okuda et al., 1982). It forms an equilibrium mixture of a hydrated five-membered hemiacetal ring structure and a hydrated six-membered hemiacetal-ring upon mutarotation (Okuda et al., 1982). Other compounds present in F5 are β -glucogallin (a simple gallotannin),

3 identified ellagitannins (Geraniin, potentillin and sanguin H-6). Compounds are possible to be identified using LCMS due to the characteristic fragments that is obtained during ionization of the compound. Fragmentation of tannins with soft ionization method will lead to loss of common fragments as highlighted in many research. Loss of common fragments of tannins include 152 a.m.u for loss of galloyl group, 301 for loss of hexahydroxydiphenoyl (HHDP) group, and 308 for loss of hexose-rhamnose sugar moiety (Salminen et al., 1999; Mullen et al., 2003; Seeram et al., 2006). Key fragments observed for tannins from LCMS analysis are fragments of m/z 633 and 301 in negative mode (Seeram et al., 2006; McDougall et al., 2008).

Confirmation of the presence of ellagitannins is upon further fragmentation of the fragment m/z 301, which results to daughter fragments of m/z 229 and m/z 257 (Mammela et al., 2000; Mullen et al., 2003; Seeram et al., 2006; McDougall et al., 2008). LC-MS/MS method was needed to distinguish between the presence of quercetin or ellagic acid as both compounds produce molecular ions of $[M-H]^-$ m/z 301 (Mullen et al., 2003). MS/MS analysis showed that further fragmentation of m/z 301 yielded characteristic daughter ions of m/z 257 and 229 which is the unique pattern for ellagic acid (Seeram et al., 2006).

In our result, Compound (**7**) showed a m/z 935.0 which was shown to be doubly charged $[M-2H]^{2-}$ by zoom scan analysis giving its real m/z as 1870 for the peak as referenced in Hager et al. (2008) as sanguin H-6 or lambertianin A. Zoom scan analysis is required to determine the charge state on an ion produced. Zoom scan data are collected using slower scans in a narrow mass range, which then improves the resolution of an analyte. This is an important tool to facilitate the correct molecular weight determination. The doubly charged deprotonated ion shows half of the m/z value for the parent ion. Doubly charged ions can be recognized by the isotopic distribution of 0.5 m/z unit separation between peaks as presented in Result. This is a common observation in analysis of large molecular weight compounds especially tannins (Mullen et al., 2003; Hager et al., 2008).

Identification of the major compound, tentatively identified as geraniin by LC-MS analysis, was then collected with a fraction collector and analyzed with NMR. Analysis with NMR is required for definitive identification of a compound. Though it is necessary, the setback of this analysis that it requires a high purity sample at a large amount especially for high molecular sample as tannins. We were only able to isolate the major compound as attempts for isolation of all the other minor compounds in F5 did not result in adequate sample for NMR analysis in terms of quantity and purity. NMR analysis of the

isolated major compound provided us with a complicated spectrum as the signals were shown for both of the isomers of the hemiacetals of the DHHD of geraniin. We then compared our shifts with shifts from previous literature. The comparison evidenced that the major compound is geraniin, as identified by LC-MS. Some of our observed shifts are showing a slightly different shift compared to the reference shift obtained from other journals. This is due to the purity of geraniin that was isolated. Although all parameters while collecting the sample are controlled to obtain the best purity of geraniin for NMR analysis, but it still does not give 100 % purity. Other compounds might still be contained in the sample for NMR analysis, thus shifting the ppm shifts of geraniin and shows a slight difference compared to the reference shift.

Geraniin shifts are identified by dissolving geraniin with acetone and few drops of d_2O (deuterated water) to provide equilibration of the two isomers. Upon equilibration, each carbon showed two peaks in the ^{13}C spectrum in a 1:1 ratio (Okuda et al., 1982). NMR has advantages in identification of tannins compounds as it provides direct information on stereochemistry and conformation of tannins, where as the disadvantage is the difficulty of interpretation of the spectra due to overlapping signals and broadening effects (Porter 1989).

The NMR spectra of hydrolysable tannins are identified to be divided to 3 regions of resonances (Porter 1989):

- 1) Resonances due to the D-glucose ring, which results in proton resonance between δ 3.5 and 6.5, and carbons shifts in the range of δ 62 to 97
- 2) Shifts from the ring protons and carbons of the galloyl moiety which occurs in the δ 6.4 to 7.2 range for the protons and δ 108 to 147 for the carbons
- 3) Peaks resonate from carbonyl of the ester in tannins, show shifts that occur between δ 165 to 170 for the carbons.

5.7 Tannins as the Bioactive Compounds in F5 of *Acalypha wilkesiana* var. *macafeana* hort. and Geraniin as the Major Compound.

One of the groups of phenolic compounds is tannins, which were named for its traditional use of 'tanning' leather by converting animal hides to leather. The ability of tannins to interact with proteins and precipitate it is its major characteristic. Tannins are also defined as water-soluble phenolic substance having molecular weights from 500 to 3000 and having a special property of ability to precipitate alkaloids, gelatin and other proteins. Most natural product

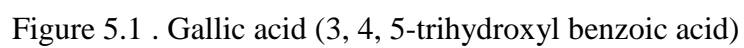
chemists define tannins to 2 classes which are hydrolysable tannins (HT's) and condensed tannins (CT's) defined by its monomer.

Environmental and geographical conditions play a role in the tannin composition of plants. These includes climate change, time of collection of the plants as well as storage conditions and drying conditions (Silva et al., 1998). Tannins *sensu lato* are present in woody plants such as *Vitis vinifera* L. (Vitaceae) and are extracted out with polar solvents such as water (Hagerman 2002). In this research, we observed that the active fraction consists of hydrolyzable tannins; 1 gallotannin (β -glucogallin) and 3 ellagitannin (potentillin, geraniin and sanguiin H-6).

5.7.1 Hydrolysable tannins (HT's)

- Gallotannins

Hydrolysable tannins are derivatives of gallic acid (3, 4, 5-trihydroxyl benzoic acid). Gallic acid is (Figure 5.1) esterified to core polyol and the galloyl groups may be further crosslinked to yield more complex HT's.



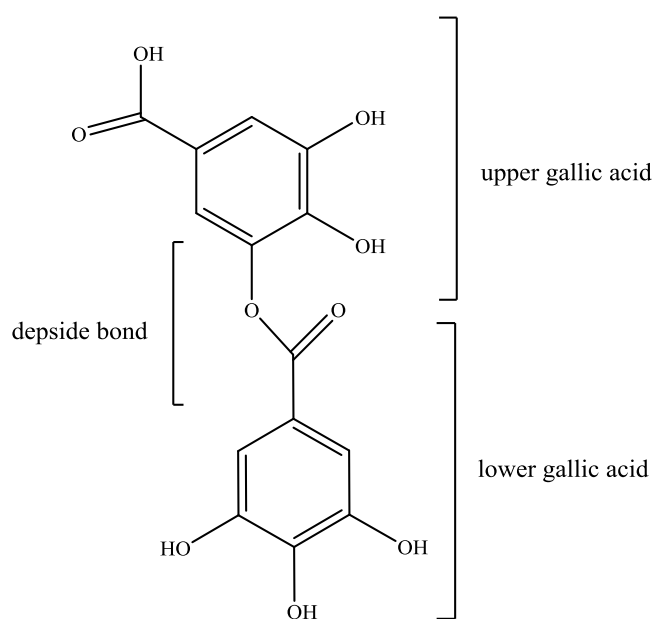


Figure 5.3. Example of a depside bond which is formed between the phenolic group of the upper and the acid group of the lower gallic acid units.

The simplest HT's are the gallotannins which are simple polygalloyl esters of glucose. A typical gallotannin would be pentagalloyl glucose which has five identical ester linkages that involves aliphatic hydroxyl groups of the core sugars (Figure 5.3). Isomers of polygalloyl ester chains are formed by either *meta*- or *para*- depside bonds, involving a phenolic hydroxyl rather than an aliphatic hydroxyl group. The depside bond is more readily hydrolyzed than an aliphatic ester bond.

- Ellagitannins

Oxidative coupling of galloyl groups converts gallotannins to the related ellagitannins. Most simple ellagitannins would be the esters of hexahydroxydiphenic acid (HHDP). HHDP spontaneously lactonizes to ellagic acid in aqueous solution (Figure 5.4).

Ellagitannins can be defined as HHDP of carbohydrates or in a wider perspective would also cover compounds derived further from oxidative transformations including oligomerization process (Okuda et al., 2009). An important keystone in understanding ellagitannins is the isolation of geraniin from *Geranium thunbergii* which was one of the most popular medicinal plants used in Japan (Okuda 1982a).

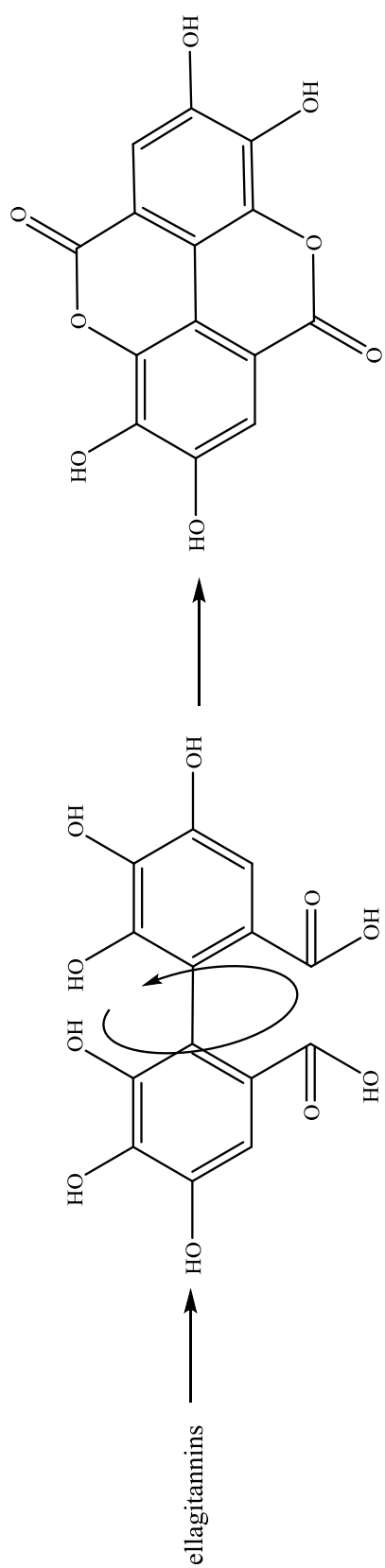


Figure 5.4. HHDP spontaneously lactonizes to ellagic acid in an aqueous solution

Occurrence of ellagitannin in plants are often monomeric or oligomeric and is the main component of a plants species (Okuda et al., 2009). The pharmacological activity of the plants is sometimes attributable essentially to that component (Okuda et al., 2009). Intramolecular carbon-carbon coupling of HHDP is most common between C-4/C-6 and C-2/C-3, as it would be more stable in a 4C_1 conformation. However, in some isolated compounds such as corilagin and geraniin, the intramolecular coupling occurs at C-3/C-6, suggesting that 1C_4 is less stable. Geraniin is further characterized by partial oxidation of the C-2/C-4 HHDP to dehydro-HHDP in aqueous solution as detected by NMR (Hagerman 2002).

5.7.2 Geraniin

The major tannin in the antibiotic fraction F5 was identified as geraniin. Geraniin is present in many plants of the Euphorbiaceae family (Okuda et al., 1982). It was first isolated from *Geranium thunbergii* Siebold ex Lindl. & Paxton, a Japanese medicinal plant used for antidiarrheic (Okuda et al., 1975). Geraniin forms an equilibrium mixture of a hydrated five-membered hemiacetal ring structure and a hydrated six-membered hemiacetal-ring upon mutarotation (Okuda et al., 1982). In this context, it is of interest to note that the family Euphorbiaceae Juss. belongs to the superorder Rosanae Takht. like Geraniaceae Juss. thus, one could reasonably draw an inference that geraniin

may very well be a major active constituent in plants of the superorder Rosanae Takht.

In fact, geraniin isolated from *Geranium sibiricum* L. (family Geraniaceae Juss.) exhibited the potent antioxidant activity together with corilagin and gallic acid (Ishimoto et al., 2012). In a recent study of polyphenol metabolites conducted with the oxygen radical absorbance capacity (ORAC) assay, geraniin and its metabolites have shown potent antioxidant activity (Ling et al., 2012). Additionally, geraniin has been found to possess apoptosis-inducing effects (Lee et al., 2008) and strong antioxidative properties in vitro (Agyare et al., 2009; Wu et al., 2010).

Besides, geraniin has been described as an anti-inflammatory compound by inhibition of TNF α -release and nitric oxide formation (Madlener et al., 2010; Kumaran & Karunakaran 2006). The latest finding has displayed geraniin as a cytoprotective agent against peroxynitrite generator 3-morpholinosydnonimine (SIN-1) and peroxy radical generator 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) in co-existence, suggesting that geraniin exhibits cytoprotective effects via free radical scavenging activity in the extracellular fluid (Ling et al., 2012). This study correlates the cytoprotective and antioxidant property of geraniin to its *ortho*-dihydroxy and galloyl groups present, as these groups are essential structures responsible for its electron

donating ability (Ling et al., 2012). A study on geraniin from *Phyllanthus amarus* recently exhibits that geraniin protects mouse liver from ethanol induced toxicity by inhibition of lipid and protein oxidation, and modulating Bax/Bcl2 ratio (Londhe et al., 2012).

Geraniin from *Phyllanthus muellerianus* (Kuntze) Exell is also identified as an effective wound healing agent as it is able to stimulate cellular activity, differentiation and collagen synthesis of human keratinocytes and dermal fibroblasts. This study isolated geraniin from *Phyllanthus muellerianus* (Kuntze) Exell (Agyare et al., 2011). The fact that geraniin possess antioxidant, cytoprotective and antibacterial properties, makes it a good candidate for an effective wound healing agent. An occurrence of wounds makes the skin exposed to external environment thus, makes it more prone to microbial attack and might delay the wound healing process (Pattanayak & Senita 2008). External application of antimicrobials to wounds prevents microbes invasion to the wound and protects it against infection (Pattanayak & Senita 2008). Research also points free radical scavenging action of antioxidants as one of the most important components of wound healing (Sen et al., 2002) and anti-wrinkle products (Cho et al., 2007).

Although geraniin is extensively suggested as a possible agent of antioxidant, antimicrobial, anti inflammatory and cancer therapy, it is observed that

geraniin are metabolized extensively in vivo with hydrolysis to ellagic acid in the small intestine being the first step (Zavala-Sanchez et al., 2011). This hydrolysis also occur in tissue culture medium, thus impose that any effects observed in cultured cellular systems may be due to geraniin or the released ellagic acid, or the combination of both. In vivo, geraniin and other ellagitannins are found to be hydrolyzed to corilagin, ellagic acid, gallic acid, brevifolin and brevifolin-carboxylic acid by gut microflora, with further metabolism to urolithins M1-M7 as presented in an excellent study by Ito and colleagues (Ito et al., 2007; Ito 2011). Whilst this evidence of metabolism of orally administered geraniin are relevant to in vivo administration, no evidence has yet been published that shows relevance of the same metabolism in other tissues.

5.8 Cytoprotection and Cytotoxic Evaluations of F5 (tannins)

The potential cytoprotective effects of both the ethanol extract and F5 against cell death induced by *t*-BHP were evaluated in HepG2 cells. It can be concluded that both the ethanol extract and F5 were non-toxic alone in all the concentration tested (6.25 – 100 µg/mL) and also protected the cells against cell death in a dose-dependent manner, with F5 being more potent than the ethanol extract.

The ability of antioxidants like ellagitannins or an extract to protect cells against oxidative insults are difficult to extrapolate only from *in vitro* chemical tests, thus *in vitro* cell culture evaluation is crucial to assess antioxidants that might be used in disease prevention and health promotion (Vidyashankar et al., 2010). Cell culture models are used to investigate biological effects of antioxidants *in vitro* as animal or human models are expensive and labor extensive (Vidyashankar et al., 2010).

HepG2 is wild-type p53 human hepatocellular carcinoma cell which is considered a good model to study *in vitro* toxicity and protection of the liver, as it retains many of the specialized functions of normal human hepatocytes (Park et al., 2010) including vulnerability to reactive oxygen species. *t*-BHP is an organic hydroperoxide which generate free radicals that initiate lipid peroxidation in HepG2, leakage of lactate dehydrogenase (LDH) and apoptosis (Yoo et al., 2008). In line with this contention, we measured protective effect of the ethanol extract and F5 against *t*-BHP-induced insults in HepG2 cells. The results obtained convincingly evidenced that F5 is cytoprotective. Since F5 consists of phenols, our results are in agreement with previous studies evidencing correlation between antioxidant capacities and cytoprotective effects (Zhang et al., 2006; Vidyashankar et al., 2010; Wu et al., 2010).

5.9 Synergistic properties of F5 (Tannin) and FESEM observation

The antimicrobial properties of tannins have been well documented by Chung et al. (1998). Tannins have been reported to be bacteriostatic (Yoshida et al., 2009) and bactericidal (Yoshida et al., 2009). Inhibitory effects of tannins are owed to the ester linkage between gallic acid and polyols (Chung et al., 1993). Scalbert (1991) summarizes the mechanism of antimicrobial action of tannins as follow:

1. Astringent character of tannin induce complexation with enzymes or substrates
2. Inhibition of electron transport system on the membranes of the microorganism
3. Complexation by metal ions.

The antimicrobial property of tannins might involve more than one mechanism above mentioned, though researches using purified tannins are still unsolved. In a review by Rios and Recio (2005), interactions with commercial antibiotics and mechanism of actions of natural antimicrobial compounds are the niche of future research that should be a more interest to researchers.

To date, evidence imply that the main mechanisms involved in tannin bacteriototoxicity is the inhibition of extracellular microbial enzymes, deprivation of nutrients, direct action on bacterial metabolism through inhibition of oxidative phosphorylation and metal ion chelation (Scalbert 1991). Ellagitannins are able to precipitate protein and remove metal cofactors through their strong affinity for metal ions, thus acting as microbial barrier (Daglia 2012).

As part of the continuous effort to identify antibiotic from medicinal plants (Wiart 2000, Wiart 2006a, Wiart 2006b, Wiart 2010), we found that *A. wilkesiana* var. *macafeana* hort. produces tannins (F5) which are bactericidal against MRSA. Synergistic studies were done to have a picture on the mechanism of action of the tannins and observe the potential of F5 to potentiate antibiotics activity. We opt to use 3 different antibiotics with 2 different mechanisms of action, which are ampicillin (inhibitor of cell wall synthesis), and tetracycline and streptomycin (inhibitors of protein synthesis). Our study demonstrates that ellagitannins from the medicinal *A. wilkesiana* var. *macafeana* hort. (F5) acts synergistically with ampicillin.

Ampicillin is a β -lactam antibiotic that functions by covalently combining with penicillin binding proteins (PBPs) and inactivating transpeptidases which are

responsible for the peptidoglycan cell wall synthesis (Zhao et al., 2001). Ampicillin was discovered in 1961 shortly after scientists from Beecham Research Laboratories found 6-aminopenicillanic acid (Rolinson & Geddes 2007). Resistance to ampicillin was soon reported in 1987, 26 years after it is introduced (Boyce et al., 1992). Currently, 100 % of *Enterococcus faecium* isolates are resistant to ampicillin in the United States (Arias & Murray 2009), though ampicillin is still prescribed for other Gram-positive infections. Search for natural product compounds which can be combined with an antibiotic for treatment of drug resistant bacteria are an important alternative to overcome bacterial resistance (Sibanda & Okoh 2010).

Studies have also unveiled the effect of polyphenolic compounds against methicillin-resistant *Staphylococcus aureus* (MRSA), and some ellagitannins were found to potentiate antibacterial effect of β -lactam antibiotics (Yoshida et al., 2009). In a study by Shiota et al. (1999), the MIC's of oxacillin, a β -lactam antibiotic, was decreased noticeably in presence of epicatechin gallate (ECG). A continuous study then observed that the effect of combining oxacillin and ECG was bactericidal against MRSA (Shiota et al., 2000). Likewise, a study of synergistic effect of corilagin and oxacillin also exhibited reduction of MIC's of MRSA with the combination (Shimizu et al., 2001).

Resistance of antibiotics in MRSA is due to the production of penicillin binding protein 2a (PBP 2a) which inactivates β -lactamase in the bacteria. The tannins were found to decrease the production of PBP 2a, inactivate PBP 2a and suppress the β -lactamase activity (Shiota et al., 2000). A series of research about compounds with synergistic ability with antibiotic, revealed that ellagitannins and condensed tannins are effective agents that reduces resistance of MRSA (Yoshida et al., 2009). The effect against the antibiotic resistance, whether it is bacteriostatic or bactericidal were found to be dependent on the polyphenolic structure (Yoshida et al., 2009).

To illustrate surface morphology of the bacterial cell wall upon treatment with the samples, FESEM analysis were performed on *Staphylococcus aureus* (ATCC 11632) and MRSA. The control cells without any treatments were full and smooth on the surfaces, as cell walls are undisrupted and well formed. Treatments with the MIC dose of the ethanol extract (1500 μ g/mL) disrupted the cell wall integrity of *S. aureus* as surface deformation was observed. MRSA cells were observed adhered to each other with biofilm forming on the cells suggesting that some amounts of intracellular materials are released as observed in Li et al. (2011). Total collapse and broken cells were observed with the treatment with MIC dose of F5 on both *S. aureus* (ATCC 11632) and MRSA, as cell debris and indentation of the cell surface were seen. This suggests total lysis of the bacteria leading to bacterial death.

5.9 Formulation of F5 and *in vivo* animal study

Formulation 1 consisted of 60 % water with a white soft paraffin and liquid paraffin base. Nature of base in which a drug is formulated has considerable effect on its efficacy and safety, thus experimenting with different excipients is extremely an important step in a formulation (Pifferi & Restani, 2003). Paraffin-water based formulation forms a hydrophobic base cream which provides an emollient effect and preferred for most end users as it is easier to apply and remove and more elegant by appearance and post application (Kanaujia et al., 2008).

The efficacy of the active tannin compounds in the formulation was observed as the test was repeated until day 10 after the formulation was prepared. Stability of the active compound in the excipients is an important parameter in formulation (Ordoñez et al., 2009). Excipients of choice have to support the active compound as in not oxidizing it or interacting with it in such a way that the efficiency of the active compound is compromised (Pifferi & Restani 2003). Stability of the active compound has to be within the recovery period of an illness so that the cream will be effective for a specific treatment (Ordoñez et al., 2009).

The ability of the active ingredient to inhibit skin against infections depends on an appropriate formulation that can maintain the integrity of the active compound and deliver it in a biological active form to the infected site (Ordoñez et al., 2009). It also has to reach the target site in sufficient quantity to be able to exert an effect and properly release bioactive substance from the carrier vehicle (Ordoñez et al., 2009). Formulation 2 (wool fat cream base) and Formulation 3 (liquid paraffin base) are unable to exert the antibacterial property wanted. This is due to the bases unable to either maintain the integrity of the tannins or unable to deliver the active compound in an active form in sufficient quantity.

Plant derived antibacterials also have a huge potential of being a topical material rather than systemic drugs and there is currently a need for new compounds of topical agents to replace current ones like fusidic acid and mupirocin where resistance have been exhibited in some instances (Gibbons 2008). Advantages considered for topical route over the systemic would particularly be the speed to market and the amount of clinical data to be approved (Gibbons 2008).

To further assess the effectiveness of Formulation 1 against *Staphylococcus aureus* infections, the formulation (dose) was tested *in vivo* on guinea pigs that

were infected with the aforementioned bacterium. It was observed that Formulation 1 promoted effective wound healing property than Burnol® as the wounds closed and healed 4 days faster, suggesting that tannins are critical in this instance. This would suggest that the antioxidant property of the formulation plays a role in the wound healing ability of the formulation. As for the antibacterial property of the formulation, it was not observed as the infections did not produce symptoms as predicted such as abscess and inflammation of the wound. This would be justified as the guinea pigs used are normal guinea pigs which have their immune defence system, that automatically prevents infections of the wounds. We would suggest future studies undergo the study with immune compromised guinea pigs in order to obtain the symptoms of the infections before treatment.

CHAPTER 6

CONCLUSION

Acalypha wilkesiana var. *macafeana* hort. was selected for the study based on its pharmacological and ethnomedicinal claims. The plant is also abundant in Malaysia, as it is an easily grown ornamental plant in tropical countries. *A. wilkesiana* var. *macafeana* hort. is identified as a traditional treatment for skin infections, wounds, inflammation and cancer, which relates to its antioxidant, antibacterial and cytoprotection properties that we have observed in our research.

Screening of the antioxidant property of the plant was assessed with Ferric reducing antioxidant power (FRAP) assay, β -carotene bleaching assay and 2,2-diphenyl-1-picryl-hydrazyl (DPPH) assay, where as the antibacterial property of the plant was tested with the pour plate disc diffusion assay. In all of the methods tested, the ethanol extract was the most active extract in both antioxidant and antibacterial assays. In view of the potential of this plant for discovery of new antioxidant and antibacterial agent for tropical countries, fractionation was commenced on the ethanol extract via chromatography affording 5 fractions. After all fractions were tested with the same assay as the

extracts, results showed that F5 was the most active fraction and warrants further study.

To shed light on the active constituents of F5, identification was done with high performance liquid chromatography (HPLC), liquid chromatography mass spectrometry (LCMS) and nuclear magnetic resonance (NMR). Separation was obtained with reversed phase HPLC which showed one major compound and 6 minor compounds. The major compound was collected with a fraction collector and identified as Geraniin via interpretation and comparison of its NMR shifts, while the other 3 compounds were identified by fragmentations of LC-MS. The compounds identified are β -glucogallin, potentillin and sanguin H-6. All identified compounds are ellagitannins, except for β -glucogallin which is a gallotannin. Three other compounds were unidentified ellagitannins without any similar fragmentation from literature.

Environment and geographical conditions plays a role in the tannin composition of plants. This includes climate change, time of collection, as well as storage and drying conditions. Tannins *sensu lato* are present in woody plants and are extracted out with polar solvents such as ethanol and water. Characterization and identification of tannins has been a challenging task due to the complexity, diversity and the large size of the compounds. Existence of multiple isomers of tannins present adds on to the challenge. With

identification of the constituents of F5, we understand that the antioxidant and antibacterial property it elicits are due to these hydrolysable tannins.

The ability of antioxidants like ellagitannins to protect cells against oxidative insults are difficult to extrapolate only from *in vitro* assays, thus *in vitro* cell culture evaluation were done to assess the cytoprotective and cytotoxicity of F5. The cell based assay was performed to HepG2 cells and F5 were observed able to protect cells against cell death induced by *t*-BHP insults in a dose-dependent manner. F5 was also found non-toxic in the concentration needed to protect the cells, which is 100 µg/mL.

We then explored the synergistic property of F5 with commercial antibiotics, as co-antibiotics are one of a new approach to overcome resistance of bacteria. We observed F5 from *A. wilkesiana* var. *macafeana* hort. and ampicillin inhibit the growth of Gram-positive bacterium synergistically. To observe the mechanism of action to the bacteria, specifically *Staphylococcus aureus*, Field Emission Scanning Electron Microscopy (FESEM) analysis was done. FESEM analysis is able to demonstrate the action of the antimicrobial agent to the bacterium surface morphology. The results that we obtained suggest that the bactericidal mechanism of F5 involves cell wall lysis as the result illustrates indentation of the cell surface and some showed total collapse of the cells.

The fact that F5 possess antioxidant, antimicrobial, and cytoprotective properties makes it a good candidate for an antimicrobial topical wound healing agent. This is explored by formulating three topical formulations containing F5, which were then tested *in vitro* against *Staphylococcus aureus*. Results pointed to formulation 1 being the effective formulation *in vitro* which consisted of water, white soft paraffin and liquid paraffin base. Nature of a base in which a drug is formulated plays a considerable effect on its efficiency and safety.

To further assess the effectiveness of the formulation, *in vivo* animal study was done on guinea pigs with an incised cut infected with *Staphylococcus aureus*. Treatment with formulation 1, Burnol® and a placebo showed that the closure and healing of the wound was faster with treatment with Formulation 1 than Burnol®. Although this is very preliminary, the result indeed gives weight to the ability of F5 from *A. wilkesiana* var. *macafeana* hort. to heal infected wounds in accordance to its traditional use.

Our results indicate possible use of ellagitannins from *A. wilkesiana* var. *macafeana* hort. with ampicillin to treat *Staphylococcus aureus* infections as it is bactericidal via a mechanism involving cell lysis. It also illustrates the possibility to be used as a topical wound healing agent with respect to its antibacterial and antioxidant properties. Taking into account its *in vitro*

bactericidal activity and the antioxidant property, the ellagitannins from *A. wilkesiana* var. *macafeana* hort. can be viewed as a new bactericidal agent that should contribute to the development of topical antibacterial drug or cosmetics or co-antibiotics in tropical countries.

In summary, our work demonstrates for the first time that the antioxidant and antibacterial properties of the plant as used in Malaysia is owed to ellagitannins such as geraniin which not only aggravates the survival of Gram-positive bacteria via cell lysis, it also fight oxidation and protect cells, thus making this Malay medicinal plant a first line candidate for the industrial pharmaceutical and cosmetic ointments for the tropical developing world.

CHAPTER 7

RECOMMENDATION FOR FUTURE STUDIES

The main purpose of this study was to contribute to the knowledge of plant species with therapeutic potential, especially in Malaysia where plants are being used in traditional medicines for various illnesses. Malaysia is a rich tropical country which encompasses of many unexplored flora. This research only covers one of the many other potential plants. Natural product research especially on obtaining justification to traditional uses and discovering new potential drug candidates should be continued.

For this specific study, recommendations for further research are described as follow:

- Identification of the minor compounds with NMR

We have identified the constituents of the active tannin fraction, F5 with fragmentation via LC-MS. As this is not a definitive method for identification, the identification remains tentative. Large scale of separation with a preparative column via HPLC should be considered in isolating the minor compounds in the fraction. Although very challenging, overcoming this barrier would open many more opportunities to understand and evaluate the every single pure compounds, not as a fraction. This might portray synergy activity

or antagonist reaction from the entire compound in the fraction, and might lead to a better result.

- Mass isolation of the major compound, Geraniin

Further understanding on geraniin as an antioxidant, antibacterial and wound healing agent would be attainable with a higher quantity of geraniin isolated. In our attempt, we managed to isolate geraniin just enough for identification via NMR. Thus, an effective isolation method could be ventured maximizing quantity, with the pure quality of geraniin that can be use to evaluate its activity in depth.

- Cytoprotection studies

Geraniin potential influence on skin cells should be assessed to validate its wound healing properties with regards to its antioxidant and antibacterial property. Cytoprotection assay can be done on dermal fibroblasts or epithelial cells in *in vitro* cell culture conditions. This might show a better understanding on its effect regarding cell differentiation, and collagen synthesis that relates to wound healing and normal metabolism of the cells.

- Geraniin as an antibacterial agent

The antibacterial activity of the tannins in F5 was only determined with 3 strains of Gram-positive bacteria, and was focussed on only *Staphylococcus*

aureus for further studies on its synergistic properties with antibiotics and SEM analysis of the surface morphology of the bacteria. We suggest that to emphasize on its ability as an antibacterial agents, tests can be performed with more Gram-positive bacteria other than *Staphylococcus aureus*. This would give a larger view and understanding of the synergistic properties, and the mechanism of action. Other commercial antibiotics with different mechanism of action should also be investigated for its synergy with the tannins such as with inhibitors of cell membrane synthesis (Polymixin B) and inhibitors of nucleic acid synthesis (Rifamycin). To make a strong case on its mechanism as an inhibitor of cell wall synthesis, synergy studies can also be performed on other inhibitors of cell wall such as Chloramphenicol.

- Formulation of the active principle as a topical medication

This study has highlighted preliminary results on the ability of the tannins from *A. wilkesiana* var. *macafeana* hort. to be formulated as a topical medication. We have determined the microbiological stability, as we tested its effectiveness against the bacteria for a certain period of time. Deeper stability studies are recommended such as physical stability evaluation, viscosity evaluation and pH measurements. Recommendation also for permeability studies of the formulation through a dermal model to validate its permeability to skin as a topical antioxidant and antibacterial agent.

- Wound healing formulation

To dwell deeper in understanding the tannins from *A. wilkesiana* var. *macafeana* hort. as a wound healing agent, we recommend including more parameters and variables such as determining the wound contraction, tensile strength, and histopathological study on the wound tissues. This would illustrate an overall understanding in the wound healing mechanism of the tannins.

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